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Institute for Genetics and Molecular Medicine

The Role of ISG15 in Cancer

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Abstract

Interferon stimulated gene 15 (ISG15) is 165 amino acids in length and its protein is roughly 15kDa. ISG15 is a member of the ubiquitin-like family (Ubl) and shows homology to several regions of ubiquitin. The ISG15 protein was first identified in 1979 in mouse Ehrlich ascites tumour cells. ISG15 is upregulated by type 1 interferons, primarily α and β . ISG15 can also be induced by IFN regulated factors (IRFs).

ISG15 has two different forms in a cell. The free form and the conjugated form. The free form has been shown to have anti-tumour effects whereas the conjugated form is generally involved in tumorigenesis. A higher concentration is found in primary tumours with the levels decreasing as the tumour progresses in stages. Therefore, ISG15 could be potentially used as an early detection diagnostic marker.

ISG15 is normally only conjugated to a small number of proteins in a cell. It has been suggested that these proteins are localized to a specific functional cellular site or else the modification significantly alters the protein. ISGylation is a post-translational modification similar to ubiquitination. ISGylation is the process through which ISG15 can be conjugated to a target protein. The ISGylation process is thought to antagonize the ubiquitin pathway. Proteins which are selected for degradation via the ubiquitin/26S proteasome pathway can be stabilised by ISGylation and avoid degradation. If this occurs with proteins which normally exert an oncogenic effect, malignant growth can ensue. This is the case with proteins such as k-Ras.

In this project, three different aspects of ISG15 were investigated; the role of type 1 interferons in ISG15 induction, targeted knock-down of ISG15 using CRISPR plasmids and single chain purification of ISG15.

To investigate the IFN induction of ISG15, cells were transfected for varying lengths of time and then the expression levels of ISG15 were compared using Western blot analysis. The optimum time of INF transfection for ISG15 production is between 16 and 24 hours with no difference being seen for transfecting for a longer time period.

The targeted knock-down of ISG15 using the CRISPR/CAS9 technique showed that the best area of the gene to target was the C-terminus as it was the only area to show a physiological change. CRISPR plasmids targeting the start of the coding exon (Exon 2) showed to have no effect.

Following the single chain purification, the activity was tested using Elisas and showed to have a high affinity for pure ISG15.

Abbreviations

BSA – Bovine Serum Albumin

Cas - CRISPR-associated

CDK1 - Cyclin-dependent kinase 1

CDRs - complementary determining regions

CPT – Camptothecins

CRISPR - clustered regularly interspaced short palindromic repeats

crRNA – CRISPR RNA

DNA – Deoxyribonucleic Acid

DSB – Double-stranded break

DTT – Dithiothreitol

EA - oesophageal adenocarcinoma

ECL – Enhanced chemiluminescence

ELISA – Enzyme-Linked Immunosorbent Assay

ESCC - oesophageal squamous cell carcinoma

Fab – Fragment Antigen Binding

Fc- Fragment Crystallisable

Fv Fragment Variable

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GFP - green fluorescent protein

HCV – Hepatitis C Virus

HF – High Fidelity

HIV - Human Immunodeficiency Virus

HNSCC - Head and neck squamous cell

HRP – Horseradish Peroxidase

IFITM - Interferon-Induced Transmembrane

IFNAR - Interferon-alpha/beta receptor alpha chain

Ig – immunoglobins

IMRT - intensity-modulated radiation therapy

INF – Interferon

iPSCs - Induced pluripotent stem cells
IPTG – Isopropyl β -D-1-thiogalactopyranoside
IRF – Interferon Regulatory factor
ISG – Interferon Stimulated Gene
ISRE - Interferon Stimulation Response Element
JAK – Janus Kinase
kDa – kilodalton
K-RAS - Kirsten rat sarcoma
mAb – Monoclonal Antibody
MHC - Major Histocompatibility Complex
MIT - Massachusetts Institute of Technology
mRNA – Messenger RNA
NCBI - National Center for Biotechnology Information
NHEJ - non-homologous end joining
NK – Natural Killer
NPC - nasopharyngeal carcinoma
OSCC - Oral squamous cell carcinoma
PAM - Protospacer adjacent motif
PBS – Phosphate- Buffered Saline
PBST – Phosphate Buffered Saline + Tween
PCR – Polymerase Chain Reaction
PDCA - pancreatic adenocarcinoma
PTEN – Phosphatase and Tension Binding
rAb – Recombinant Antibody
RIG - Retinoic Acid-Inducible Gene
RNA – Ribonucleic Acid
RNAi – Interference RNA
scFv – Single-Chain Variable Fragment
SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis
sgRNA - Single-guide RNA

siRNA – silencing RNA

STAT - signal transducer and activator of transcription

SUMO - Small Ubiquitin-like Modifier

TAE - Tris base, acetic acid and EDTA

TALEN - Transcription activator-like effector nucleases

TLR – Toll-Like Receptor

TRAIL - TNF-related apoptosis-inducing ligand

trancrRNA - trans-activating crRNA

TRIM25 - Tripartite motif-containing 25

UBE1L - Ubiquitin E1-like protein

UBE2E - Ubiquitin-conjugating enzyme E2

USB18 - Ubiquitin specific peptidase 18

VEGF - Vascular endothelial growth factor

V_H – Variable heavy chain

V_L – Variable light chain

VRE – Virus Response Element

WT – Wild Type

XIAP - X-linked inhibitor of apoptosis protein

ZNF - zinc finger nucleases

Chapter 1:

Introduction

Interferon stimulated gene 15 (ISG15) is an interferon-regulated protein. It is 165 amino acids in length and is roughly 17kDa.(Tao et al. 2015; Li et al. 2014) (Dao et al. 2006) ISG15 is a member of the ubiquitin-like family (Ubl) and shows homology to several regions of ubiquitin. (Wood et al. 2012; Desai et al. 2006a) Other UBLs include NEDD8, SUMO1-4 and ATG8 & 12. The ISG15 protein was first identified in 1979 in mouse Ehrlich ascites tumour cells by Farrell and colleagues by using ubiquitin antibodies. (J et al. 2015; Pitha-Rowe & Pitha 2007) It was first noted as a ubiquitin cross-reactive protein ISG15 is one of the most highly induced ISGs.

1.1 Interferons and INF regulated genes

Interferons are divided into 3 categories; type 1 interferons (INF α , β , ω and τ), type 2 interferons (INF γ) and type 3 interferons (INF λ). (Jeon et al. 2010) ISG15 is upregulated by type 1 interferons, primarily α and β .

1.1.1 Type-1 Interferons

Type 1 IFNs operate through the activation of the Janus Tyrosine Kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway. (T. Li et al. 2013; Marotta et al. 2011) Interferons are members of the cytokine family and as such the help regulate the innate immune response to target infections and tumour cells. (Sainz et al. 2016) Type 1 IFNs play a very important role in the innate immune response. (Liu et al. 2004; Katsounas et al. 2013) They are involved in inhibiting cell cycle progression and promoting programmed cell death and so they have been used in leukaemia treatments as well as other cancers. (Kim et al. 2015) The release of IFNs signals the start of a signalling pathway that ends in the upregulation of interferon-stimulated genes. (Andersen & Hassel 2006)(Liu et al. 2011)

The presence of interferons has been noted in multiple serious viral infections such as HIV, hepatitis and human papillomavirus. (Katsounas et al. 2013) Despite this, hepatitis is the only one of these viral condition to utilize type-1 IFNs in treatment options. (Geffin et al. 2013) There have been mixed reports on the use of Type-1 IFNs in HIV therapy as well as in the treatment of other chronic viral infections. (Harman et al. 2011)

1.1.2 IFN Regulated Factors

ISG15 can also be induced by IFN regulated factors (IRFs). (Juang et al. 1998) The 55 kDa IRF 3 protein was the first IRF to be reported. It can be activated by viral infection. (Desai et al. 2006) (Juang et al. 1998) The ISG15 gene has a 5'-cis-regulatory sequence called the interferon-stimulated response element (ISRE). Several IRFs such as IRF 3 and IRF 9 can form a bond with the ISRE for ISG15 induction. (Desai et al. 2006b; Kim et al. 2015). ISG15 can be induced through many pathways however. ISG15 is targeted by NF- κ B and by p53. The crucial genes regulated by IRF1 were identified by Harman and colleagues through micro-array expression. (Harman et al. 2011)

1.1.3 Interferon- Induced Transmembrane Proteins:

First identified by Brass and colleagues, interferon-induced transmembrane (IFITM) proteins are powerful suppressors of many viral infections such as West Nile virus. It also has the ability to suppress HIV-1 replication. (Lu et al. 2011) Members of the IFITM protein family contain two transmembrane domains as well as a highly conserved intracellular region. The N-terminus and the C-terminus face away from the plasma membrane toward the extracellular environment. (Lu et al. 2011) (Liu et al. 2011) IFITM1 is also known as 9-27 or Leu13. IFITM1 is induced by INF γ which is a downstream member of the ISGylation pathway. IFITM1 is stimulated by interferons due to the presence of an interferon stimulation response element (ISRE) in their promoters. (Dao et al. 2006) IFITM proteins are involved in cellular mechanisms such as cell adhesion, oncogenesis as well as immune response. Tumours can also show elevated levels of IFITM proteins. (Lu et al. 2011) (Liu et al. 2011) Despite their important role in cellular functions, IFITM1 knockouts do not show embryonic lethality. (Liu et al. 2011)

Following an infection, type 1 interferons are induced. (Forster 2012) (Dieterich & Relman 2011) (Katsounas et al. 2013) In cells, this results in an activation of many pathogen-recognition receptors. (Forster 2012) Interferons are induced by the activation of the IFN regulatory factor (IRF) family members. IRFs are stimulated virally and are activated through either TLR3 endosomally or through the RIG-1 like helicase family member which is cytoplasmically located. (Forster 2012)

IFITM proteins are encoded by ISGs and display similar antiviral activity. (Lu et al. 2011) IFITM proteins display their antiviral activity by obstructing the entry of the virus into the cell.

This is due to the fact that IFITM proteins are membrane associated and block viral entry through membrane mediated inhibition. (Lu et al. 2011)

1.2 Functions of Free ISG15

ISG15 can be present in a cell as free ISG15 or in a conjugated form. (Fan, Miyauchi-Ishida, et al. 2015)(Burks et al. 2015) Free ISG15 and conjugated ISG15 exhibit very different effects. Free ISG15 stimulates the immune systems and provides anti-viral and anti-tumor protection. Conjugated ISG15 utilizes the ISGylation pathway to bind to substrate proteins. (Burks et al. 2015; Liu et al. 2004; Desai et al. 2006a) Free ISG15 can be released extracellularly and intracellularly. Extracellular ISG15 displays cytokine-like functions, (Jeon et al. 2010) Extracellular ISG15 was first identified in 1991 after human lymphocytes and monocytes released ISG15 after IFN β transfection. It stimulates the production of type II IFNs, activate NK cells and induce non-major histocompatibility complex-restricted cytotoxicity of tumour cell targets.(Fan, Arimoto, et al. 2015) (Fan et al. 2015) When ISG15 is secreted into the extracellular matrix it tends to have an antitumour function by inducing NK cell proliferation and migration towards the tumour. (Scagnolari et al. 2016) (Liu et al. 2004) It also induces the release of IFN γ from lymphocytes. In ISG15 knockout mice the lack of NK cell migration is fatal in embryos. (Scagnolari et al. 2016) As free ISG15 invokes an innate immune response it stands to reason that humans with an inherited ISG15 deficiency are more prone to bacterial disease than their homozygous counterparts. (Desai 2015) A lack of intracellular ISG15 disrupts the aggregation of USP18 which is a strong negative regulator of type 1 interferon signalling. This causes an augmentation of INF α and INF β response. (Chinyengetere et al. 2015a) (Falvey 2016a) A severe increase in INF α and INF β causes an upregulation of extracellular ISG15 and an unnecessary overactive immune response. (Andersen & Hassel 2006)

ISG15 in the intracellular matrix activates MHC-Class 1 antigen presentation, from the adaptive branch of the immune system. Therefore, both forms ISG15 seem to create a very robust defensive system against cancer. (Falvey 2016a)

1.3: ISGylation and Conjugated ISG15

ISGylation is a post-translational modification similar to ubiquitination. ISG15 was initially named ubiquitin cross-reactive protein as it was binding to the same antibodies as ubiquitin. (Andersen & Hassel 2006) ISGylation is the process through which ISG15 can be conjugated

to a target protein. ISGylation functions in a similar yet non-identical fashion to ubiquitination. (Fan, Arimoto, et al. 2015)

1.2.1 Members of the ISGylation Pathway:

The sequential action of enzymes E1, E2 and E3 are necessary to transfer ISG15 to its target protein. (Desai et al. 2006a) There are a variety of enzymes in the E1, 2 and 3 family. Ubiquitin E1-like protein (UBE1L) is the ISG15s activating enzyme. E1 is an ATP-dependent enzyme which is responsible for activating the pathway. (Pitha-Rowe & Pitha 2007) (Jeon et al. 2010) Ubiquitin-conjugating enzyme E2 (UBE2E) is the conjugating enzyme necessary for ISGylation. Interferon-induced transmembrane protein (IFITM1) contains remnants of the active site of E2 and so it has been theorised that it expedites the E2 function by creating an isopeptide bond between ISG15 and its targeted protein. (Fan et al. 2015) Tripartite motif-containing 25 (TRIM25) is an example of an E3 ligating enzyme. (Liu et al. 2011) UBE1L can form a thioester bond with ISG15 yet it does not with ubiquitin. This indicates that it is specific to ISG15 ISGylation. UBE1L's C-terminal has a ubiquitin fold domain that is necessary for the transfer of ISG15 to UbcH8 from UBE1L. (Desai et al. 2008a)

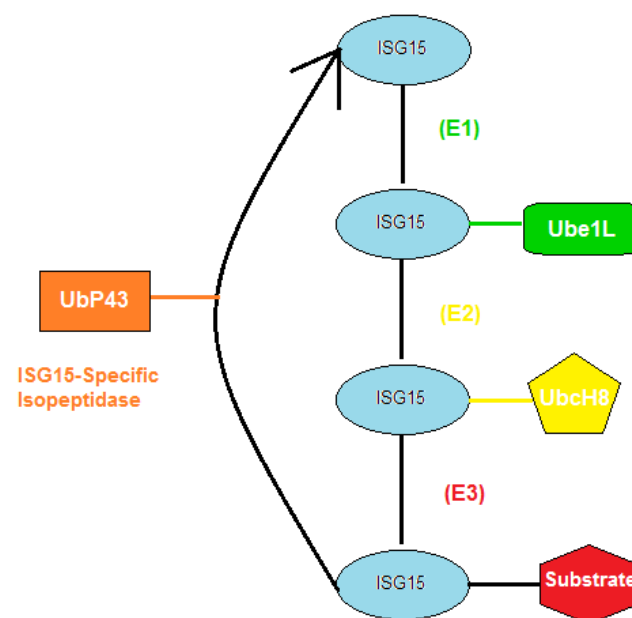


Figure 1.1: Image shows the sequence enzymes needed for ISG15 conjugation to a targeted substrate

USP18 (UBP43) is an ISG15 regulated enzyme and is involved in the stabilisation of antiviral proteins. USP18 is also known to suppress INF α and drug induced programmed cell death by binding to IFNAR2 and displacing JAK1. (Li et al. 2014) This supports the theory that some ISGylation genes which are involved in antiviral defence can also have chemo-protective functions when associated with oncogenesis. (Liu et al. 2004) The effects of ISGylation are two-fold; firstly, the direct addition of ISG15 alters the protein, secondly, it antagonizes the ubiquitin pathway preventing the degradation. ISG15 is normally only conjugated to a small number of proteins in a cell. A question which has often been raised is how such a small percentage of ISGylated proteins can have such a significant biological effect. It has been suggested that these proteins are localized to a specific functional cellular site or else the modification significantly alters the protein. (Desai 2015)

1.2.2 ISGylation and the Ubiquitin Pathway

The ISGylation process is thought to antagonize the ubiquitin pathway. Proteins which are selected for degradation via the ubiquitin/26S proteasome pathway can be stabilised by ISGylation and avoid degradation. (Asher et al. 2005) If this occurs with proteins which normally exert an oncogenic effect, malignant growth can ensue. This is the case with proteins such as k-Ras. The exact mechanism through which the ISGylation pathway antagonizes has not been fully elucidated yet there are several logical theories. It is believed ISGylation may disrupt the pathway by monopolising the E1, E2 and E3 enzymes which are needed in both processes. The pathways also compete for substrate binding sites. ISG15 binds to substrates through its UBL domains thus it binds to the same sites on the target protein as ubiquitin. (Wood et al. 2012) Lys 29 is an ISG15 specific binding site on ubiquitin. ISG15 can also bind to NEDD8 and SUMO modification site, Lys 48. (Fan et al., 2015). Free ISG15 has the ability to bind to NEDD4 and blocks NEDD4 from mediating ubiquitination. (Liu et al. 2004; Wood et al. 2012) NEDD8 conjugates to a member of SCF-E3 ubiquitin ligases, Cullins, which initiates the enzymatic activity of E3 ligases and aids the conjugation of ubiquitin to its cellular targets. (Jeon et al. 2010) A recent report has also put forward a third branch of inhibitory functions. A paper by Fan and colleagues in 2015 described a process in which ISG15 actually binds to ubiquitin itself forming mixed-chains. ISG15 can bind to ubiquitin, incorporating itself into the ubiquitin chains which affects ubiquitin's ability to degrade targeted proteins. Proteasome inhibitors alter the amount of ISGylated proteins within a cell. (Desai et al. 2012; Desai et al. 2006b) As ISGylation is seen as an antagonist of the ubiquitination process the

level of polyubiquitinated proteins decreases as the level of ISGylated proteins increases. (Wood et al. 2012)

When UBE2L6 is silenced in cells, the cell becomes more responsive to chemotherapy and are more apoptosis competent. (J et al. 2015) The same results are not seen when ISG15 is knockdown using siRNA. This leads us to believe that the apoptotic effect of UBE2L6 is separate to the ISGylation pathway. (J et al. 2015; Liu et al. 2004)

Proteomic investigations have shown that ISG15 itself has the ability to alter ubiquitin. It does this by assimilating into ubiquitin chains which has an adverse effect on the degradation of ubiquitylated proteins. (Fan, Miyauchi-Ishida, et al. 2015)

The ISGylation process targets over 300 substrate proteins in human cells. The proteins involved in this process include proteins involved in cytoskeleton organization, RNA splicing, stress response and translation. (Fan, Miyauchi-Ishida, et al. 2015) Many proteins that form parts of an antiviral signalling pathway are targets for ISGylation. (Chinyenetere et al. 2015b) In order to understand the role of ISG15 conjugation, one first needs to identify the proteins that undergo ISGylation. One of the most valuable tools used for identifying ISG15 conjugated proteins is the 293T conjugation system. This method involves ISG15 modifying a small amount of the protein in question. This protein can then be detected after affinity enrichment. (Desai et al. 2012)

1.3 ISG15 in Cancer:

ISG15 was first discovered in 1979 yet very little research was completed around that time. (Desai et al. 2006a) It was only when ISG15 was seen to be deregulated in cancers that some serious investigation was undertaken. From this ISG15 roles such as its antagonizing the ubiquitin pathway and its involvement in cell survival were discovered. (Laljee et al. 2013)

1.3.1 Anti-tumour Effects of ISG15:

As previously stated ISG15 has two different forms in a cell, the free form and the conjugated form. The free form has been shown to have anti-tumour effects whereas the conjugated form is generally involved in tumorigenesis. (Desai 2015)

It is possible that free ISG15 functions differently in different parts of the cell. In the extra cellular milieu, it may function to establish antitumour innate immune response by activating

NK cells in vivo. (Burks et al. 2015) Intracellular free ISG15 may contribute to activate the adaptive arm of the immune system by enhancing MHC-class 1 antigen presentation in vivo. Vaccination against free ISG15 results in CD8-mediated reductions in both primary and metastatic mammary tumour in mice. (Desai et al. 2012) Tumour cells deregulate the function of free ISG15, probably by blocking its secretion by conjugating it to cellular proteins consequently, escaping immune surveillance. (Chen et al. 2016)

Due to its presence in a variety of cancers, ISG15 is quite rapidly becoming used as an oncogenic biomarker. (Falvey 2016a)

1.3.2 Pro-tumour Effects of ISG15:

ISG15 stabilizes proteins via the ISGylation process. When this process is upregulated, proteins which should be degraded escape ubiquitination. (Fan, Miyauchi-Ishida, et al. 2015; Dao et al. 2006) Ubiquitin maintains the homeostasis of cellular proteins and so when this balance is altered aberrant changes begin to happen within the cell. (Desai et al. 2012) ISG15 is known to bind to K-Ras and Survivin, both of which are known oncogenes. It has been proposed that ISG15 modulates Survivin ubiquitination via XIAP. (Li et al. 2014) The presence of ISG15 weakens the link between XIAP and Survivin. ISG15 is believed to block XIAP from interacting with Survivin which leads to Survivin being stabilized. (Li et al. 2014) (Y. R. Li et al. 2013) ISG15 can reduce expression of Cyclin B1 and CDK1 and can induce G2/M phase cell cycle arrest. It can also boost tumour cell migration. XIAP functions as an active centre of E3 and is responsible for the ubiquitination of substrates. (Li et al. 2014)

Due to the fact that cancer treatments can increase the level of ISG15 conjugates, ISG15 is thought to be a tumour suppressor. However, an overexpression of ISG15 is correlated oncogenesis. (Desai et al. 2008b) When conjugated, ISG15 loses its immunological ability. It is the disruption of the ISGylation pathway that is associated with tumour progression. Increased levels of ISG15 conjugates have been detected in many tumours. A higher concentration is found in primary tumours with the levels decreasing as the tumour progresses in stages. Therefore, ISG15 could be potentially used as an early detection diagnostic marker. (Burks et al. 2015)

1.3.3 ISG15 and the Cancer Stem Cell Niche:

The tumour-associated niche of many solid tumours provide information to a subpopulation of tumour-initiating cells: cancer stem cells. (Bibikova et al. 2006) (Sancho, Alcala, et al.

2016) These stem cells provide the signals and cues necessary for self-renewal and metastatic activity. Sainz et al showed in their 2014 paper that ISG15 is found in pancreatic adenocarcinoma (PDCA) tumour cells. PDACs secrete $\text{INF}\beta$ which upregulates ISG15. (Sainz et al. 2014) ISG15 in turn creates the stem-like environment that amplifies the oncogenic properties of the tumour. This could be due to the stabilizing of proteins that generally increase the 'stemness' of a cell. K-ras is one of Yamanaka's key transcription factors which was used in his experiment to create iPSCs. (Okita et al. 2007) K-ras is a known oncogene and is also one the proteins which is stabilized by ISGylation. ISG15 deficiency suppresses K-ras driven lung tumorigenesis and ISGylation of p53 may contribute to tumourigenesis. (Li et al. 2014)

An ISG15 deficiency silences ki-Ras dependent tumour formation in lung tissue. ISGylation bolsters the oncogenic effects of ki-Ras that induces tumorigenesis in vivo. (Zhang et al. 2015)

1.3.4 Cancer Fieldisation:

Cancer fieldisation or cancer landscape is a newly discovered phenomenon in cancer research. In this process, the tumour and the surrounding tissue have numerous mutations yet something triggers the malignant growth in the tumour. (Stransky et al. 2011)(Simple et al. 2015) The unusual thing about this phenomenon is that in a lot of cases there is a higher number of mutations in the tissue surrounding the tumour than in the actual tumour. This suggests that there is a specific mutation in the tumour cells which initiates tumorigenesis. This has been seen in squamous tissue cancer of the oesophagus. (Gaykalova et al. 2014) The niche provides growth factors and cell signalling cues which promotes the oncogenic nature of the tumour and its metastatic ability. (Simple et al. 2015)(Sancho, Alcala, et al. 2016)

1.3.5 ISG15 in Head and Neck Cancer:

Head and neck squamous cell cancer (HNSCC) has a significant mortality rate. With 560,000 new diagnoses a year and 300,000 deaths, it makes up 5% of all tumour patients. The survival rate is as low as 50% within 5 years after diagnosis. (McBride et al. 2014) This type of cancer is largely related to risk factors such as smoking and high alcohol consumption yet cases can occur without partaking in any of these habitual factors. (Stransky et al. 2011a) (McBride et al. 2014) Despite advances in many other malignancies, treatments for HNSCC have remained stagnant over the past few decades. The current treatment is the use of chemotherapy in conjunction with intensity-modulated radiation therapy (IMRT). (Gaykalova et al. 2014) Like most cancers, HNSCC develops after a build-up of multiple genetic and epigenetic mutations. Several distinctive steps occur in this process such as the allelic loss of 9p, 3p, 17p, 11q, 3q,

14q, 6p, 8p and 4q. This results multiple mutations in genes such as CDKN2A, TP53 and pTEN. (Stransky et al. 2011a)

Oesophageal cancer is the 8th most common malignancy with a mortality rate of almost 5%. (Yan et al. 2012) There are two main histological types; oesophageal adenocarcinoma (EA) and oesophageal squamous cell carcinoma (ESCC). In ESCC, surgery is the most common form of treatment as it provides the only possibility of a complete cure. It has a 5-year survival rate of greater than 95% if the entire tumour is resected in its early stages. (Tao et al. 2015) ESCC is difficult to detect in early stages however and so when the cancer is discovered the tumour has generally progressed to an advanced stage and the prognosis is poor. (Bektas et al. 2008) Excessive alcohol consumption is a major risk factor for ESCC and is associated with a poor survival rate. A higher expression of ISG15 mRNA was detected in ESCC tissues compared with non-tumour tissue samples from the same patient. The expression of ISG15 mRNA increases as the stages progress so it has been used as a potential marker for a worse prognosis. (Tao et al. 2015) (Bektas et al. 2008)

Oesophageal cancer has one of the worst prognosis of any cancer in the western world. In Europe, it has a survival rate of under 15% and is the 8th most common cancer worldwide. (Falvey 2016b) ISG15 plays an important role in oesophageal cancers. It is overexpressed in roughly 80% of OSCC tissue samples. (Falvey 2016a) Components of the ISGylation pathway are upregulated in the cancer cell lines which are apoptosis proficient and show a positive response to chemotherapy. (Yan et al. 2012) (Falvey 2016a) ISG15 conjugates to substrates that are otherwise soluble. This changes the secretome of the oesophageal tissue creating an anti-tumour effect. (Falvey 2016)

1.3.6 ISG15 in Breast Cancer:

ISG15 stabilizes key cellular proteins in breast cancer cells. It stabilizes proteins involved in cell migration/metastasis conferring increased motility and promotes breast cell transformation. Blocking ISG15 or UbcH8 reverses this transformed phenotype. (Marotta et al. 2011) ISG15 silenced tumours grow rapidly compared to ISG15 overexpressing tumours in nude mice. Recombinant free ISG15 inhibits tumour growth when added extracellularly and induce intratumour infiltration of NK cells in nude mice. Intracellular free ISG15 enhances 26S proteasome-dependent surface expression of MHC class I complex on breast cells. (Burks et al. 2015) (Desai et al. 2012)

ISG15 has been shown to alter focal adhesions and cytoskeletal proteins. (J et al. 2015)

Breast cancer cell lines which exhibit invasive tendencies also show higher levels of ISGylated proteins. Correspondingly, non-invasive cell lines showed much lower levels of these proteins. (J et al. 2015; Burks et al. 2015) ISG15 can play a negative role in breast cancer. As the ISG15 pathway antagonizes ubiquitination and prevents the degradation of cellular proteins that are associated with cell migration. This causes the cancer cells to have increased motility as well as promoting breast cell transformation. (J et al. 2015)(Zhang et al. 2015)

1.3.7 ISG15 in Nasopharyngeal Cancer:

ISG15 is differentially expressed in nasopharyngeal carcinoma. (Zhou et al. 2015) In NPC tissues, elevated amounts of ISG15 corresponds to a shorter overall survival as well as disease-free survival. This is likely due to the fact that ISG15 overexpression creates a cancer stem cell phenotype such as pluripotent gene expression and increased tumorigenesis. (Zhou et al. 2015) (Sancho, Barneda, et al. 2016) ISG15 knockdown abated the stem cell characteristics in NPC cells. (Sancho, Barneda, et al. 2016) An overexpression of ISG15 in INC cells conferred resistance to the chemotherapeutic drug cisplatin as well as to radiation. (Zhou et al. 2015) (Chen et al. 2016) For these reasons ISG15 can be used as a prognostic marker for NPC. Work is also being carried out to utilise ISG15 as a therapeutic target. (Chen et al. 2016)

1.3.8 ISG15 and Camptothecin:

Camptothecins (CPT) e.g. topotecan are chemotherapeutic drugs used as a treatment in a variety of cancer types. (Liu et al. 2004) Tumours are known to show a varied level of resistance to camptothecin. ISG15 was shown to be a determinant for CPT resistance. Knock-down of ISG15 or UbcH8 resulted in an increased proteasomal degradation of the CPT-induced TOP1-DNA covalent complexes. (Desai et al. 2008a) (Desai 2015) CPT destroy tumour cells by capturing topoisomerase 1 – DNA covalent complexes. The ubiquitin/26S proteasome pathway is induced by CPT which degrades the TOP1-DNA covalent complexes. This highlights the importance of the ubiquitin pathway in CPT resistance. (Liu et al. 2004) (Jeon et al. 2010)(Jeon et al. 2012) Tumours with high levels of ISG15 will not respond positively to camptothecin, therefore treatment of tumours with a high ISG15 content should have tailored chemotherapeutic treatment plans. (Desai et al. 2008a)

IFNs and JAK/STAT are dispensable for camptothecin-mediated induction of ISG15. The ISG15 conjugates which are generated by camptothecin are different from the conjugates

which are produced by type 1 IFNs. (Wood et al. 2012) Camptothecin significantly increases the IFN-induced ISGylation of proteins. Treatment of colorectal cancer in mice with a mixture of IFNs and camptothecin shows synergistic cell death. (Desai et al. 2008a)(Liu et al. 2004)

1.4 ISG15 in Viruses:

ISG15 and its conjugates hinder viral replication in vivo. Congruently some viruses initiate viral specific proteins that have the ability to deconjugate ISG15 from its conjugates which initiates an antiviral response. (Domingues et al. 2015) In UBE1L $-/-$ mice the ISGylation of proteins does not occur. ISG15 $-/-$ mice show no obvious signs of abnormalities and also have the ability to reproduce. Nor does a lack of ISG15 affect the cells immune system. (Eduardo-Correia et al. 2014) $INF\alpha$ and $INF\beta$ have been used as clinical drugs since their discovery in the mid-20th century. $INF\alpha$ has been used to treat chronic cases hepatitis B and C virus as well as certain cancers such as leukaemia. $INF\beta$ was shown to be effective at treating multiple sclerosis. (Pitha-Rowe & Pitha 2007) (Broering et al. 2016)

ISGylation has been shown to suppress Ebola through blocking ubiquitin ligase NEDD4. Nedd4 is required by viral budding. Although many proteins can be targeted by ISGylation, ISG15 tends to target newly synthesized host and viral proteins.(Broering et al. 2016) This process allows for localised antiviral activity without the need for widespread protein modifications in the cell. (Liu et al. 2011)

1.4.1 ISG15 and HCV:

ISG15 has a multitude of functions within the cell. A number of ISGs have been reputed to suppress HCV replication. (Katsounas et al. 2013)The exact mechanisms have not been fully elucidated yet early reports show the viral replication is blocked by ISG action at several steps in the HCV replication cycle. (Pitha-Rowe & Pitha 2007) The recent literature portraying the role of ISG15 in HCV is contradictory yet the popular opinion veers on the side of a proviral effect. When ISG15 is overexpressed HCV, replication increases yet when it is silenced it inhibits HCV replication to a relatively low extent. (Domingues et al. 2015)

1.4.2 ISG15 and HIV:

An induction of ISG15 is seen when the Human Immunodeficiency Virus (HIV) is inhibited by type 1 IFNs. (Scagnolari et al. 2016) Moreover, when ISG15 is overexpressed it copies the effect of IFN and suppresses the release of HIV-1 virions. In HIV-1 provirus cells, the

overexpression of ISG15 and UBE1L completely inhibit HIV replication. (Katsounas et al. 2013) UBP43 expression however can rescue HIV replication. As the expression of ISG15 alone fails to block viral replication, it must therefore be the ISGylated proteins and not ISG15 itself that blocks the viral replication. (Scagnolari et al. 2015; Katsounas et al. 2013)

Type 1 interferons have multiple capacities with regards to HIV-1 infection. The viral increase that is associated with HIV infection results in an augmentation in interferon α due to HIV's ability to counteract the antiviral activity of interferons. (Scagnolari et al. 2015)

Members of the ISGylation pathway, including ISG15, can activate the production of indoleamine 2,3-dioxygenase which controls TNF-related apoptosis-inducing ligand (TRAIL). (Harman et al. 2011) These are known to apply immunosuppressive effects on T-cells. The debilitated immune system seen in HIV patients is likely due to this mechanism. (Geffin et al. 2013)

1.5 The CRISPR – Cas Process:

Type II prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR)/Cas adaptive immune system is capable of aiding RNA-guided site specific DNA cleavage. (Cong et al. 2013)(Sander & Joung 2014) CRISPR and CRISPR-associated (Cas) proteins are part of a bacterial immune system which can detect and kill any invading microorganisms. (Domingues et al. 2015) This method has recently been adapted for the generation of genetically engineered cells or organisms.

The CRISPR loci are comprised of an array of repeats that are distanced by 'spacer' sequences which are identical to the genome of bacteriophages. This repeat-spacer series is transcribed in a long precursor and is processed to create short CRISPR RNA that determine the target sequences cleaved by CRISPR. (Gaj et al. 2013) Protospacer adjacent motif (PAM) is a sequence motif which is directly downstream of the target region and is necessary for cleavage. (Cong et al. 2013)

1.5.1 Cas9 Targeting

Cas 9 nucleases are directed by short RNAs in order to produce a precise cut at a genomic locus in eukaryotic cells.(Wang et al. 2013; Sander & Joung 2014) Cas9 may also be changed into a nicking enzyme to aid homology-directed repair with the least amount of mutagenesis. (Hwang

et al. 2013) The Cas genes generally flank the repeat-spacer series and contain the essential enzymatic equipment required for genome targeting. (Wang et al. 2013) Cas9 is comprised of a double-stranded DNA endonuclease which utilizes the crRNA guide to determine the cleavage site. (Domingues et al. 2015) Cas proteins, CRISPR RNAs and transacting crRNA form ribonucleoprotein complexes that target and destroy foreign nucleic acid. This complex is guided by the crRNAs. The cas9 endonuclease can be programmed to produce sequence-specific DSB if a manufactured single-guide RNA containing crRNA and tracrRNA is provided. (Jiang et al. 2013) A lot of work has been done to determine the nucleases whose sequence specificity is designed by small RNAs. (Jiang et al. 2013) Single-guide RNA (sgRNA) is an amalgamation of crRNA and tracrRNA and can direct Cas-9 cleavage of target DNA. This cleavage can also be induced by crRNA and tracrRNA functioning together. (Jiang et al. 2013) (Sander & Joung 2014)

1.5.2 Benefits of CRISPR:

Traditional genome editing techniques have included the use of creating mutations through homologous recombination in embryonic stem cells of mice. (Gaj et al. 2013) These cells must then be injected into the blastocysts of wild type mice which will then generate chimeric offspring. This method is arduous and time consuming and extremely costly to knock-out just a single gene. New methods to speed up this process have since been under development. (Wang et al. 2013) The DNA of site-specific nucleases can be injected directly into the one-cell embryo which can generate a double-stranded break at a specific site. The DSB is then repaired by error-prone non-homologous end joining (NHEJ). (Wang et al. 2013) (Cong et al. 2013) Targeted integrations can occur if a donor plasmid with corresponding ends to the DSB is coinjected. This requires zinc finger nucleases (ZNF) or transcription activator-like effector nucleases (TALENs) specific for each gene. The CRISPR-Cas system is an advancement of this process. (Gaj et al. 2013)

The CRISPR Cas system is a much cheaper and less time consuming alternative for genome editing. It can quickly and effectively knock down multiple genes in a single cell. In a 2013 paper written by Wang et al, the authors describe the process of knocking down 5 separate genes in a single mouse model simultaneously. This paper shows the dynamic effect of the CRISPR Cas system in large scale gene editing. Efficient genetic adaptation is necessary for the analysis of disease and development. A CRISPR array can contain several guide sequences

that can enable simultaneous editing of several sites. This shows the ease at which the mammalian genome can be edited by using CRISPR. (Wang et al. 2013)

1.5.3 CRISPR Design Process:

To design CRISPR oligos, programmes such as MIT Optimised CRISPR design program are used. This program scans the target genomic region and produces multiple possible designs for the short RNA which guides the Cas9 enzyme. The RNA chosen should have the least off-target sites. The MIT program shows a score out of 100 for each short-RNA with the highest score depicting those RNAs which have the fewest off-target sites. An oligo with a score of over 90 should be chosen.

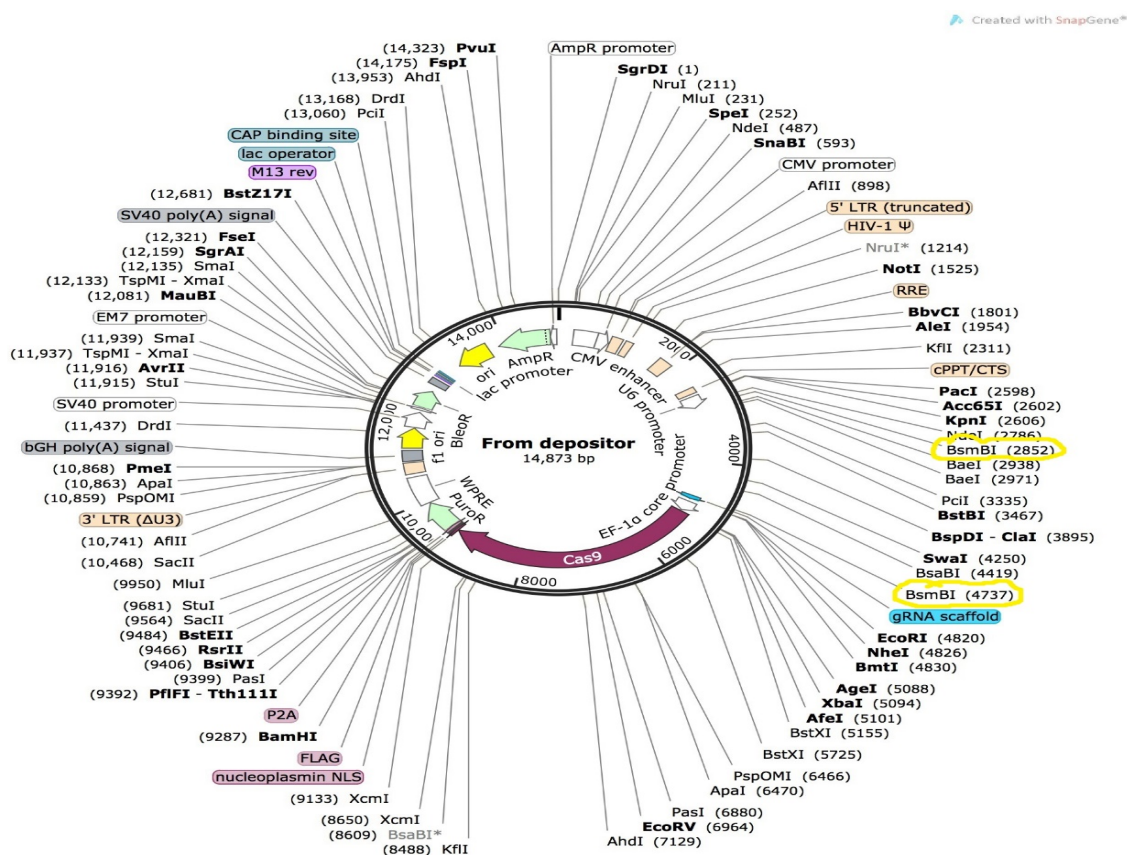


Figure 1.2: Sequence map of the plasmid used in the plasmid digestion.

Image by SnapGene

Once a short-RNA design has been chosen, it must be incorporated into a viral plasmid in order to be transfected into the cell. Lentiviruses are often used as they can be economically sourced and are easy to manipulate. In the lentiviral plasmid there are two BsmB1 sites which are 1885 bp apart. Using the BsmB1 restriction enzyme the vector can be cut at these two sites leaving

a linear plasmid. A double digestion can be carried out in order to ensure that the plasmid has been cut efficiently. The cut vector can be digested with a restriction enzyme for BamH1. If the vector has been cut by the BsmB1 restriction enzyme the BamH1 enzyme will bisect the cut linear vector and we should get two separate bands. If the BsmB1 restriction enzyme did not cut the vector then when the BamH1 restriction enzyme cuts, we should get one linear plasmid which would appear as just one band on a gel. Once the linear plasmid has been created the short-RNA can be integrated into the plasmid so that the viral plasmid contains the short-RNA oligo.

1.6 Single Chain Purification:

Antibodies are of great importance in biological research, specifically the antibodies that show high specificity and high affinity. Their importance stems from their ability to quantify gene expression levels, identify specific loci of gene expression as well as determining interactions between the protein and other molecules. (Farajnia et al. 2014)

1.6.1 Classic Antibodies

Antibodies are also known by the term immunoglobins (Ig). An IgG is a bivalent, Y-shaped antibody. Monoclonal antibodies (Mabs) have some limitations when it comes to biomedical research and so antibody fragments were created in order to surpass MAb limitations. (Hoet et al. 2005) Previously, these limitations had been overcome by eliminating the Fc region by proteolytic treatment with enzymes such as pepsin. General antibodies are comprised of 4 polypeptide chains two of which are heavy and two of which are light. The light chains contain roughly 220 amino acid residues and the heavy chains are approximately double this at 440 amino acid residues. (Moestrup & Gliemann 1989) (Huston et al. 1993) (Farajnia et al. 2014) These chains then fold into domains of roughly 110 amino acids that form a conserved 3D conformation. These domains can then associate into specific structural areas. The antigen binding region, also known as the variable region (Whitlow et al. 1993) is created by the interaction of the amino termini of the heavy and light chains. (Huston et al. 1988)

1.6.2 Single Chain and Peptide Linker Composition

In recent years the need for more specific antibodies has led to the development of recombinant antibodies composed of immunoglobulin variable region genes. The most useful of these recombinant antibodies is the single-chain Fv antibody. (Malpiedi et al. 2013) ScFv fractions

are the smallest V_H - V_L molecules that are capable of high affinity antigen binding. The single chain Fv contains the entire binding site of the antibody in a small, single polypeptide chain. The binding site of the scFv can reproduce the affinity and specificity of the original antibody combining site. The molecular weight of the polypeptide is roughly 26,000.

ScFv is comprised of antigen-binding domains of Ig heavy (V_H) and light (V_L) chain regions linked by a flexible peptide. All these components are encoded by a single gene. These single chain antibodies are highly useful for in vivo studies where conventional techniques had previously failed. (Huston et al. 1993) The V_H and V_L genes can be obtained from a specific hybridoma cell line, taken from a V-gene library or genetically synthesised. (Smith 1985)

A single chain is formed when the two V gene components are joined using an oligonucleotide which encodes specific linker peptides, Gly-Ser for example. The linker peptide joins the beginning of the V region to the N-terminus of the second. (Whitlow et al. 1993) Different linker peptides are used for different specificities. Glycine and serine linkers are used for flexibility and glutamic acid and lysine charged residues are used for improved solubility. Linker peptides are usually between 15 to 20 amino acid residues. (Whitlow et al. 1993) The binding site of the antigen is formed by the joining of the two V chains. Within the antigen binding domain of immunoglobulin there are three hypervariable regions which are known as complementary determining regions (CDRs). These form loops and display high sequence variability and responsible for the recognition of the antigen. (Hoet et al. 2005)

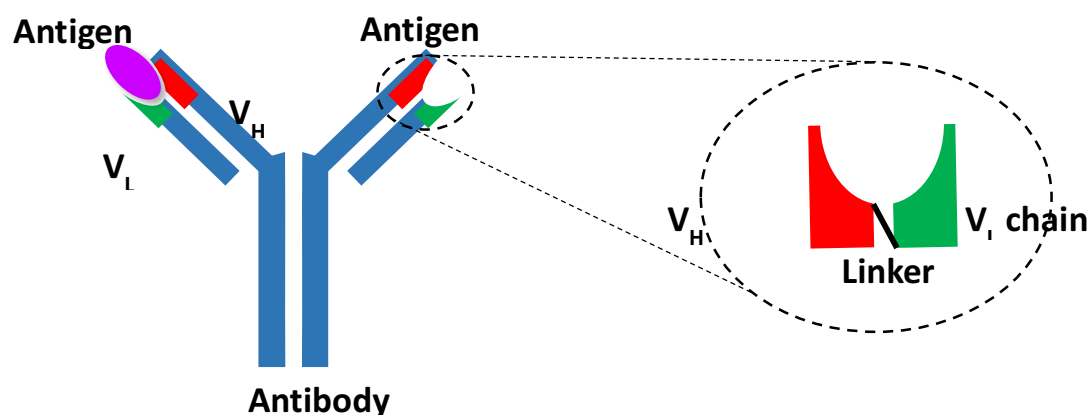


Figure 1.3: Figure depicts the composition of an antibody to that of the single chain components.

1.6.3 Phage Display and Library:

For the phage display, the antigen-binding segments of V_H and V_L genes are cloned and used to manufacture scFv gene stores. (Sheets et al. 1998) These stores are then cloned as fusion proteins with a minor coat protein of bacteriophage to create a phage antibody library. Each phage from the library will have an operational antibody protein on its surface. They also contain the gene which encodes the antibody. (Casey et al. 1995) Certain phage antibodies that bind explicitly to proteins can be sorted apart from phage antibodies that fail to bind through affinity chromatography techniques. The quantity and affinity of antibodies created for a specific antigen is directly proportional to the size and assortment of the library. This poses problems as the production of large libraries has proven difficult. (Casey et al. 1995; Sheets et al. 1998)

The phage display system was first introduced in 1985 when Smith and colleagues displayed that foreign DNA fragments can be fused to the gene encoding the phage minor coat protein of a bacteriophage and consequently expressed in the form of a fusion protein at the surface of the phage without affecting its sensitivity. (Smith 1985) This technique was then utilised by professors in Cambridge to produce single chain fragments on the surface of the virion that are capable of binding to antigens. (Sheets et al. 1998)

To select a scFv from phage display libraries, numerous rounds of probing against the antigen required is undertaken with washing steps completed inbetween to remove nonspecific phage clones. In general, many rounds of probing with increased washing inbetween is needed to ensure only high affinity binding phages. (Malpiedi et al. 2013) Using this technique, scientists were able to create a high-throughput screening system for phage display that aids antibody identification and characterization. (McCafferty et al. 1990) For the single chain purification an elution step is needed in order to retrieve the phage molecules that contain the scFv fractions which are able to bind to the target protein. Low pH buffers are commonly used in the elution phase. (Smith 1985)

1.6.4 Benefits of Single Chain Antibodies:

scFvs have many positive qualities. The high stability of the phages allow them to be stored at 4°C for as long as needed and its production is highly economical as it only requires infecting *E. coli*. (Huston et al. 1988) ScFvs have become increasingly important as forms of cancer research and cancer treatments. They possess the ability to recognize specific markers present on the cell surface of tumours. The reduced size of the fragments also allows for rapid bio-

diffusion. (Lillo et al. 2011) The rapid infusion is considerably faster than that of IgG or FAB which allows the imaging of isotopes with a short half-life.

These single chains can be developed to recognize any antigen required. They can also be designed specifically for certain protein domains as well as for the different epitopes in the domains. Immunological therapies are fast becoming some of the most important treatments in cancer therapy. Antibodies can be created to recognize antigens on the surface of tumour cells. Examples of this include anti-Her2 scFv and anti-VEGF ScFv. (Chen et al. 1999) The *Escherichia coli* expression system is the most popular choice for recombinant ScFv production. (Schmidt et al. 2001)

Monoclonal antibodies can be ineffective in multiple biological techniques however as accidental activation of the effector functions can cause toxicity due to cytokine release. Single chain fragments can be much more effective in biological processes. (Hoet et al. 2005)

Aims

The aim of this project was three-fold. First we aimed to investigate the role of type 1 interferons in the induction of the ISG15 pathway. Secondly we aimed to investigate the knock down of ISG15 in cells using the CRISPR Cas system. Lastly we aimed to induce and purify and single chain antibody which had a high affinity for ISG15 protein. These aims combined will give us a deeper insight into the composition and function of ISG15.

Chapter 2

Materials and Methods:

Materials:

Solutions Used:

PBST (1L)

10x PBS – 100ml

ddH₂O – 900ml

Tween – 1ml

Urea Lysis Buffer (2ml)

25mM Hepes pH 7.5 - 50μl

7M Urea – 840mg

25mM NaCl - 25μl

10% Triton x-100 - 10μl

5mM DTT - 10μl

ddH₂O – 1.905ml

1 M HEPES (1L) pH 7.5

Dissolve 238.3 g HEPES in 800 mL of ddH₂O

Adjust the pH to the desired value with 10 N NaOH

Bring up the volume to 1 L with ddH₂O

50x TAE (1L)

Dissolve 242.2 g Tris base in around 600 mL of ddH₂O

Slowly add 57.1 mL glacial acetic acid

Add 100 mL 0.5 M EDTA, pH 8

Bring up the volume to 1 L with ddH₂O

10x DNA loading buffer (100 mL)

Measure 20 mL 50x TAE into a 100-mL graduated cylinder

Add 40 g sucrose

Add 10 mg bromophenol blue

Bring up the volume to 100 mL with ddH₂O

Ampicillin (1000x)

Dissolve 5 g ampicillin in 25 mL ddH₂O

Add 25 mL absolute ethanol

Store at -20 °C

Running Buffer (1L) 10x

30g Tris

144g Glycine

10g of SDS

1000ml of H₂O

Dilute to 1X before use

10x Transfer buffer (4L)

121.1 g Tris base

576 g glycine

Bring up the volume to 4 L with ddH₂O

1x Transfer Buffer (1L)

Methanol – 200ml

Transfer Buffer 10x – 100ml

ddH₂O -700ml

1M Tris pH 9 (50ml)

6.6g of Tris

Dissolve in 40ml of ddH₂O

Bring pH to 9 using drops of HCL

Bring up to 50ml with ddH₂O

0.2M Glycine pH2 (50ml)

0.75g of glycine

Dissolve in 25ml of ddH₂O

Bring pH to 2 using drops of HCL

Bring up to 50ml with ddH₂O

PBS-Triton 1% (40ml)

4ml of PBS 10X

4ml of 10% Triton x100

32ml of ddH₂O

Coomassie Blue Solution (1L)

Dissolve 1 g of Coomassie Brilliant Blue (Bio-Rad) in 1 litre of the following solution:

Methanol (50% [v/v])

Glacial acetic acid (10% [v/v])

H₂O (40%)

Stir the solution for 3-4 hours and then filter through Whatman filter paper. Store at room temperature.

Destaining Solution

50% ddH₂O

40% Methanol

10% acetic Acid

Native Gel

1.5M Tris pH 8.8

1.0M Tris 6.8

30% Acrylamide

10% SDS

10% APS

Computer Programs Used:

Microsoft Word

Microsoft Excel

Microsoft Powerpoint

Image Studio Lite

Antibodies Used

Swine Anti-Rabbit 2 λ 7

Rabbit Anti-Mouse 260

GAPDH mAb ab9484

Abcam Rabbit ISG15 polyclonal Antibody

Mouse Anti-IRF1 antibody

Til anti-IFITM1 antibody

Protein A HRP

Laboratory Machine Equipment:

Centrifuge 5415R Eppendorf

Perkin Elmer Lambda Bio

Soniprep 150 PLUS

Perkin Elmer Multilable Counter

Li-Cor Odessey Scanner

Syngene Bio-Imaging System

Bio-Rad Power Pack

Nanodrop 2000c Spectrophotometer

Biorad Tetrad 2 PCR Thermal Cycler

Cell Media

Sigma FBS

Gibco Penicillin Strip

Gibco RMPI 1640 Media

Siha Cells – Cervical Cancer Cells

Kits Used:

Quiagen Gel Extraction Kit

Quiagen PCR Purification

Quiagen Miniprep Kit

Amaza Cell Line Nucleofactor Kit T

Miscellaneous Reagents and Equipment

Thermo Scientific PageRuler Plus Prestained Protein Ladder

Cellulose Nitrate Membrane Filter

Attractene

ISG15 Gene Transcript:

Exon 1:

CGCAGGCTCGGCGGCACGCCCCCTGACGTGTGTGCCTCAGGCTTATAATAGGGCCGGTGC
TGCCTGCCGAAGCCGGC**GGCTGAGAGGCAGCGAACTC**ATCTTTGCCAGTACAGGAGCTTG
TGCCGTGGCCACAGCCACAGCCACAGCCATG

Intron 1 & 2: 407

Exon2:

GGCTGG**GACCTGACGGTGAAGATGCT**GGCGGGCAACGA**ATTCCAGGTGTCCCTGAGCA**GC
TCCATGTTCGGTGTTCAGAGCTGAAGGCGCAGATCACCCAGAAGAT¹**CGGCGTGACGCCTTC**
CAGCAGCGTCTGGCTGTCCACCCGAGCGGTGTGGCGCTGCAGGACAGGGTCCCCCTTGCC
AGCCAGGGCCTGGGCCCCGGCAGCACGGTCCTGCTGGTGGTGGACAAATGCGACGAACCT
CTGAGCATCC**TGGTGAGGAATAACAAGGGC**CGCAGCAGCACCTACGAGGTACGGCTGACG
CAGACCGTGGCCACCTGAAGCAGCAAGTGAGCGGGCTGGAGGGTGTGCAGGACGACCTG
TTCTGGCTGACCTTCGAGGG
GAAGCCCCTGGAGGACCAGCTCCCGCTGGGGGAGTACGGC
CTCAAGCCCCTGAGCACCGTG²**TTCATGAATCTGCGCCTGCG**GGGAGGCGGCACAGAGCCT
GGCGGGCGGAGCTAAG

UTR

GGCCTCCACCA**GCATCCGAGCAGGATCAAGG**GCCGGAATAAAG
GCTGTTGTAAAGAGAAA

CRISPR RNAs

Exon 2 CRISPR RNAs:

Forward: [Phos]CACCG¹AGGCGTGCACGCCGATCTTC

Reverse: [Phos]AAACGAAGATCGGCGTGCACGCCTC

C-Terminus CRISPR RNAs:

Forward: [Phos]CACCG²TTCATGAATCTGCGCCTGCG

Reverse: [Phos]AAACCGCAGGCGCAGATTCATGAAC

PCR Primers

Short (Original) Primers:

Forward: GACCTGACGGTGAAGATGCT

Reverse: GCCCTTGTTATTCCTCACCA

TGGTGAGGAATAACAAGGGC

Medium Primers:

Forward: ATTCCAGGTGTCCCTGAGCA

Reverse: CCCTCGAAGGTCAGCCAGAA

TTCTGGCTGACCTTCGAGGG

Long Primers:

Forward: GGCTGAGAGGCAGCGAACTC

Reverse: GCATCCGAGCAGGATCAAGG

CCTTGATCCTGCTCGGATGC

Methods:

Preparing Cell Lysate

Remove all media from plate using the aspirator. Wash three times with 4°C PBS. Add 1ml of 4°C PBS. Scrape cells using a cell scraper and move to an Eppendorf tube. (All above steps to be carried out on ice) Spin for 5 mins at 3000rpm at 4°C. Discard supernatant (Pellets can be flash frozen at this point) Resuspend in X μ l of lysate buffer depending on size of pellet. Leave on ice for 30 mins. Centrifuge at 13,200rpm for 15 mins. Remove supernatant and place in freshly labelled Eppendorf tubes. Test concentration using Bradford reagent.

Bradford Assay

1 μ l of each sample being tested was added to 200 μ l of Bradford Reagent in a clear microplate. 1 μ l of the lysate buffer used was added to 200 μ l of Bradford Reagent to act as a control. 1 μ l of a titration of BSA standards of known concentrations were also added to 200 μ l of Bradford Reagent. All of these samples are blanked against the cell lysate well. The concentrations of the unknown samples are calculated using the known samples are standards.

Quantitative Protein Measurements

5 μ l of all samples (known and unknown) are added to 5 μ l of loading dye. These samples are run on an SDS-PAGE gel and then stained using Commassie Blue stain. The gel is destained thoroughly using destaining solution. The gel is rehydrated with deionised water for 15 minutes or until the has returned to its original size. The gel is scanned using the Odessey gel Scanner. The intensity of the bands is then calculated using the Image Studio Lite Gel Analysis program. The intensity levels of the known samples are mapped on an excel graph and the concentrations of the unknown samples are calculated based on the equation of this graph. BSA standards are used as the known samples.

Running a Western with Cell Lysate

Prepare a gel with the correct percentage for the protein you are trying to identify and prepare running buffer. Add roughly 30ng of protein to each lane. Amounts will vary with different concentration levels. Run gel at 180V for 1hr. Transfer at 100v for 1hr or 30V overnight. Optional: Stain with ink for 15 mins – 1 drop of ink in 15ml of PBST while shaking. Wash three times with 15ml PBST while shaking for 5/10 mins. Block with 5% PBST milk for 30mins-1hr while shaking. Add primary antibody for 1hr. (If left overnight dilute in 15ml of

5% PBST milk on the shaker in cold room) Wash three time with 15ml PBST while shaking for 5/10mins. Add secondary antibody 2.5ml of 5% PBST milk for 1hr. Wash three times with 15ml PBST while shaking for 5/10 mins. Develop using ECL.

Developing using ECL

Create your ECL solution by adding equal parts of ECL1 and ECL2 to a falcon tube. Total volume of 2ml is sufficient for 1 blot. (Solution must be mixed directly prior to use) Add solution directly to blot and leave for 1 min. Blot off excess solution using absorbent paper. Attach blot to glass plate with cling film. Develop for periods of 5 seconds, 10 seconds, 5 minutes and 10 minutes. Draw in ladder on developed blot.

PCR Protocols:

Before starting the PCR process, the primers must be tested to determine their optimum temperature. This is usually 3°C higher than then lowest temperature primer. DNA must be extracted from the wild type cell using the Quiagen DNA extraction kit.

Total Volume: 20µl HF Phusion Protocol

2x Phusion HF: 10µl

10µM Forward Primer: 0.1µl

10µM Reverse Primer: 0.1µl

DNA: 250ng

DMSO: 0.6µl

ddH₂O: Variable

Running Sequence:

Step	Temperature	Time
Initial Denaturation	98°C	30 Seconds
	98°C	10 Seconds
Annealing	Variable (45°C -72°C)	Variable (depends on length between primers)
	72°C	10 Seconds
Final Extension	72°C	10 Minutes
Hold	4°C	Forever

PCR Purification Protocol for Sequencing

PCR products are purified using the Qiagen Purification Kit. The concentration of the purified product is calculated using the nanodrop machine.

Freezing Cells

Samples are trypsonised for 5 minutes. The trypsonisation reaction is stopped by adding 10ml of media. The cells are then spun down at 1000rpm for 5 minutes. The supernatant is removed and the pellet is resuspended in 3ml of freezing media. 1ml of the sample is aliquoted into cryotubes and placed in a freezing pack. The freezing pack is placed at -80°C for one week. Following this the cryotubes can be removed from the freezing pack and placed in normal storage boxes at 80°C. When the cells are needed again, the cryotubes should gently thawed on ice. When fully defrosted the 1ml of defrosted cells is added to 9ml of media in a P₁₀₀ plate. The media on this plate is changed after 24 hours. The cells should not be split for at least 48 hours as they will be quite sensitive.

Plasmid Design:

To design my CRISPR guide RNA I used the MIT optimizer website. This website scans the desired genome for guide sites and gives them a score based on their number of off-site targets. A high score (>90) is preferred. The website generates a 20bp oligo based on these criteria. Bases were then added to either end to ensure smooth ligation into the plasmid. The reverse oligo is designed based on the generated forward oligo. These oligoes must be annealed before they are ligated.

Plasmid Digestion:

Cut the lentiviral CRISPR vector using the BsmB1 restriction enzyme (NEB). The restriction enzyme is 10000u/ml. 1 unit digests 1µg. 10µg of DNA is needed for digestion which is the equivalent to 1530ng/µl as the concentration of DNA is 600ng. Therefore, 1µl of restriction enzyme was used.

Vector used = 16.6 µl

Enzyme (BsmB1) = 1µl

Buffer 3.1 (10X) = 5µl

De-ionised H₂O = 27.33

The reaction was carried out in a heating block at 55°C for 1hr in a total volume of 50µl. Following this the mixture was heated at 80°C for a further 20 minutes. Following this 1µg of phosphatase enzyme was added and left at 37°C for 1hr.

(A phosphatase reaction is needed in order to remove the phosphate group on either end of the cut plasmid in order to prevent them from re-circularizing. This will ensure that the guide RNA can anneal properly.)

Gel Purification of Digested Plasmid

To make a 1% agarose gel measure out 1g of agarose and mix it with 100ml of 1 X TAE buffer. Heat in the microwave for approx. 4 mins, stirring occasionally. Allow it to cool at room temperature. When it has sufficiently cooled safe green is added at 1:10000. Finally pour it into the apparatus and allow to cool and set for at least an hour. Before running the gel, a loading buffer must be added to the DNA mixture. Run the gel at the 100v for 30-45 minutes. After the gel has run use a clean sharp scalpel to cut your DNA at the 12KB band. This band should be the 13KB cut vector. The DNA can now be extracted using the Quiagen Gel Extraction Kit.

Annealing the pairs of Oligos:

The forward and the reverse strands of the guide RNA must be annealed together to form a double stranded DNA insert that can be ligated with the backbone of the cut plasmid. To do this there must be equal concentrations of the forward and reverse strands and a 10X T4 ligation buffer. This experiment should be carried out at 10µl so to make up the final concentration we add ddH₂O.

1 µl forward strand (100 µM)

1µl of reverse strand (100µM)

1µl of 10X T4 ligation buffer (NEB)

7µl of dd H₂O

The tube is place in a water filled hole in the heat block at 95°C for 5 mins. Following this the heating block is then switched off but the tube remains in it and is allowed to cool down to room temperature slowly.

Ligation Reaction:

For the ligation reaction there must be a defined molar relationship between the insert and cut plasmid. The most common ratios are 1:0.5, 1:1, 1:2 & 1:3. The reaction takes place at 10µl so 100ng of the backbone plasmid is needed. The plasmid used was 75ng/µl and so 1.25µl was used to make up the concentration. The concentration of insert was calculated based on ration as well as other factors such as vector size, vector ng and insert size. This calculation was carried out using the *INSILICO* ligation calculator. http://www.insilico.uni-duesseldorf.de/Lig_Input.html

	1:0.5	1:1	1:2	1:3
<i>T4 Ligase (µl)</i>	1	1	1	1
<i>T4 Buffer 10X (µl)</i>	1	1	1	1
<i>Backbone Plasmid (µl)</i>	1.25	1.25	1.25	1.25
<i>Insert (µl)</i>	0.328	0.657	1.309	1.971
<i>ddH₂O (µl)</i>	6.422	6.093	5.441	4.779

The tubes were kept at 16°C over night.

Transforming the Competent Cells:

Add 2µl of each of the ligation reaction to 50µl Stb3 cells. Incubate for 30 minutes on ice. Following this heat shock at 42°C for 60 seconds. Return the tubes to ice for 3 minutes then add 50µl of LB to each tube. Incubate the tubes for 1 hour in the 37°C shaker. After this inoculate agar plates with two concentrations of each ligation ratio; 10µl and 50µl. Incubate these place at 37°C overnight.

Plasmid sequencing:

15µl of each plasmid ratio being sequenced was sent along with 5µl of the primer per tube. Plasmid concentration was 100ng. To achieve the correct concentration, the plasmid was

diluted using EB buffer. 2µl of plasmid was added to 17µl of EB buffer and the Nano Drop was used to analyse the new concentration. Using this as a base small amounts of EB or plasmid were added to achieve the correct concentration. P.F. primer that has a concentration of 3.2µM was used. A concentration of 0.1µM was needed and so 1.6µl of the primer was added to 48.4µl of H₂O. The promoter used was U6.

Nano drop

The nanodrop can be used to test genomic DNA concentrations. 1µl of the buffer the DNA is diluted in is placed on the nanodrop and blanked. 1µl of the DNA sample is placed on the nanodrop and its concentration is recorded. This is repeated 3 times and an average concentration of the DNA is calculated.

Double Digestions:

Double digestion can be used in order to ensure that the plasmid has been effectively cut by the BsmB1 restriction enzyme leaving a 2KB deletion in the plasmid (it cuts in two places). In this double digestion the restriction enzymes BamH1 and BspD1. BspD1 has a restriction site in the segment that should have been cut and so this should not have any effect of on the plasmid if it has been effectively cut. The second digestion enzyme used was BamH1. This restriction enzyme has a site in the 13KB plasmid and so it should cut the plasmid into two segments, one roughly 5KB and the other 8KB. This can be shown when run on an agarose gel.

	Plasmid 1	Plasmid 2	Plasmid 3	Plasmid 4	Full Lentivirus Plasmid
<i>BamH1 (µl)</i>	0.25	0.25	0.25	0.25	0.25
<i>BspD1 (µl)</i>	0.5	0.5	0.5	05	0.5
<i>10X Cut Smart (µl)</i>	4	4	4	4	4
<i>Plasmid (µl)</i>	10	5	5	5	2
<i>ddH₂O (µl)</i>	25.25	30.25	30.25	30.25	33.25

Plasmid Transfection:

The plasmids were cotransfected with GFP for FACS cell sorting.

4mg of Plasmid

4mg of GFP

15 μ l of Attractane

Variable amount of free media (Media with no FBS or pen strip)

Total Volume: 300 μ l

The wild type Siha cells were transfected when they were roughly 60% percent confluent and then they were sorted 48 hours later when they were roughly 90-100%.

Electroporation:

Three p100 plates of 90% confluent Siha cells were washed and trypsonised as usual. Media was then added up to 10ml. All the media was added into a 50ml falcon and was spun down at 1000rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in 2ml of media. 10 μ l of cells and 10 μ l trypsin blue were added together and 10 μ l of this solution was placed in a cell counter slide and the number of cells were calculated using the cell counter. 1 million cells were needed for the electroporation and so based on the number of cells present I calculated that I needed 390 μ l of the cell media. This 390 μ l was placed in an Eppendorf and was spun at 300g for 7 minutes. The supernatant was removed and the pellet was resuspended in 100 μ l of Cell Line Nucleofactor Solution T, 2 μ g (6.5 μ l) of plasmid and 1.2 μ g (3 μ l) of GFP from the electroporation kit. (The following steps must be undertaken quickly as the Nucleofactor is toxic to cells) This solution was added to a cuvette and placed in the Nucleofactor Device and the program V-23 for high expression was selected. Once the program had finished I added 500 μ l of media to the cuvette. This was then transferred to 1.5ml of media that had been previously incubated in a 6-well plate. I added 200 μ l (0.5 million cells) to an adjacent well and made up to 2ml with media to act as a control. These cells will be left for 90hrs before FACS cell sorting. The media on the cells were changed 48hrs later to remove the dead cells.

FACS Cell Sorting:

Before cell sorting the cells are trypsonised as usual. Media is added to stop the reaction and the cells are added in a fresh 15ml falcon tube. They are spun at 1000rpm for 5 minutes. The supernatant is removed and the pellet is resuspended in 0.2ml of PBS.

2 x 96 well plates containing 200µl of media and one falcon tube containing 2ml of media are prepared per sample.

Single Chain Purification:

A sterile tip was used to scrape the starter culture (plasmid in a glycerol stock). This tip was placed in a falcon tube containing 5ml of fresh LB, 10% Amp and 0.1% glucose. (Glucose is used to stop expression of the plasmid before transfection i.e. leaky expression) This was left shaking at 37°C overnight. The following day this 5ml was added to 250ml of fresh LB with 10% amp and 0.1% of glucose. This was left shaking at 37°C until the OD600 levels were between 0.4 and 0.6. The OD level was checked after 1hr and then every 20 minutes after that until the OD600 level was at 0.555. This took roughly 2.5hrs. 1ml of this solution was spun down at 13,200rpm for 15mins at 4°C and frozen in liquid nitrogen. This sample will be run on a commassie gel later.

Plasmid Induction Protocol A:

I then added 250µl of 1M IPTG to the rest of the solution and left it shaking at 30°C overnight. The next morning the flask contents were diluted 1:1 with fresh LB and the OD600 absorbance levels were measured at 1.621 and 1.747 respectively. A 1ml aliquot was taken, spun down at 13,200rpm at 4°C for 15mins and frozen in liquid nitrogen. This sample will be run on a commassie gel later. The rest of the sample was spun down at 4000rpm at 4°C for 40mins. The pellet was then resuspended in 10ml of PBS.

Plasmid Induction Protocol B:

I added 250µl of 1M IPTG to the rest of the solution and left it shaking for 6hrs at 37°C. I checked the OD600 level every hour and took a 1ml aliquot and spun it down at 13,200rpm at 4°C for 15 mins. After the 6 hours I spun the rest of the sample down at 4000rpm at 4°C for 40 minutes. The supernatant was discarded and the pellet was resuspended in 10ml of PBS.

Plasmid Induction Protocol C

I added 250µl of 1M IPTG to the rest of the solution and left it shaking for 3hrs at 37°C. I checked the OD600 level every hour and took a 1ml aliquot and spun it down at 13,200rpm at 4°C for 15 mins. After the 3 hours I spun the rest of the sample down at 4000rpm at 4°C for 40 minutes. The supernatant was discarded and the pellet was resuspended in 10ml of PBS.

Purification of the plasmid

100µl of 10% Triton x100 is added and the tube is placed on the rotator in the cold room for 30mins. Following this the tube was sonicated 6 x 15 seconds with a 30 second interval inbetween or until the sample has a consistency of water. The solution is then decanted into 1.5ml Eppendorf's and spun down at 13,200rpm for 15 minutes at 4°C. The supernatants (cell lysate) were collected together and filtered twice, once through a 45µM filter and once through a 22µM filter. A 20µl aliquot was taken and frozen in liquid nitrogen. This sample will be run on a commassie gel later. 50µg of protein A Sepharose Resu powder was added to 600µl of ddH₂O and was dissolved by gently inverting the tube. Using the lowest centrifuge setting the Eppendorf is spun for 30 secs three times, discarding the supernatant inbetween. 550µl ddH₂O was added to the beads and this was then added to the cell lysate. This mixture was then placed on the rotating wheel in the cold room for 1hr. Following this the mixture was added to a 10ml column and the flow-through was collected and frozen in liquid nitrogen. After the flow-through was collected the beads were washed 5 times with 1ml of a PBS+1% triton solution. These washes were collected and frozen in liquid nitrogen. The single chain was then eluted with 500µl of 0.2M glycine pH2. The elutes were collected in a tube which contained 75µl of 1M Tris pH 9. Five elutes were collected in total and they were kept at 4°C.

All of the sample aliquots taken, the flow-through, the washes and the elutes were run on 12% SDS PAGE gels. The gels were then stained using commassie blue stain for 35 minutes and then destained using destaining solution overnight.

Elisa Protocol:

Coat 20 wells of an ELISA plate with 100ng of protein in 50µl carbonate mix. Coat 10 control wells with 50µl of just carbonate mix. Leave overnight at 4°C. Wash 5x with PBST. Blocked for 1hr in 100µl of 3% BSA-PBST. Variety of dilutions were used as the primary antibody. Primary antibody was left shaking for 1hr. Wash 5 x 200µl PBST. Secondary was added at a

dilution 50 μ l per well and left shaking. Wash 5 x 200 μ l PBST. 50 μ l of ECL I and ECL II mixture was added to each well and developed using fluorescent machine.

Dot Blot Protocol:

2 μ l of protein is added to a membrane. Membrane is blocked in 15ml of 5% PBST-Milk for 1hr. Primary is added for 1hr. Wash x3 PBST. Secondary is added for 1hr. Wash x3 PBST. Develop with ECL mixture

Chapter 3

Results:

3.1 Interferon Induction of the ISG15 Pathway

3.1.1 *INF α* induction Vs. *INF β* induction

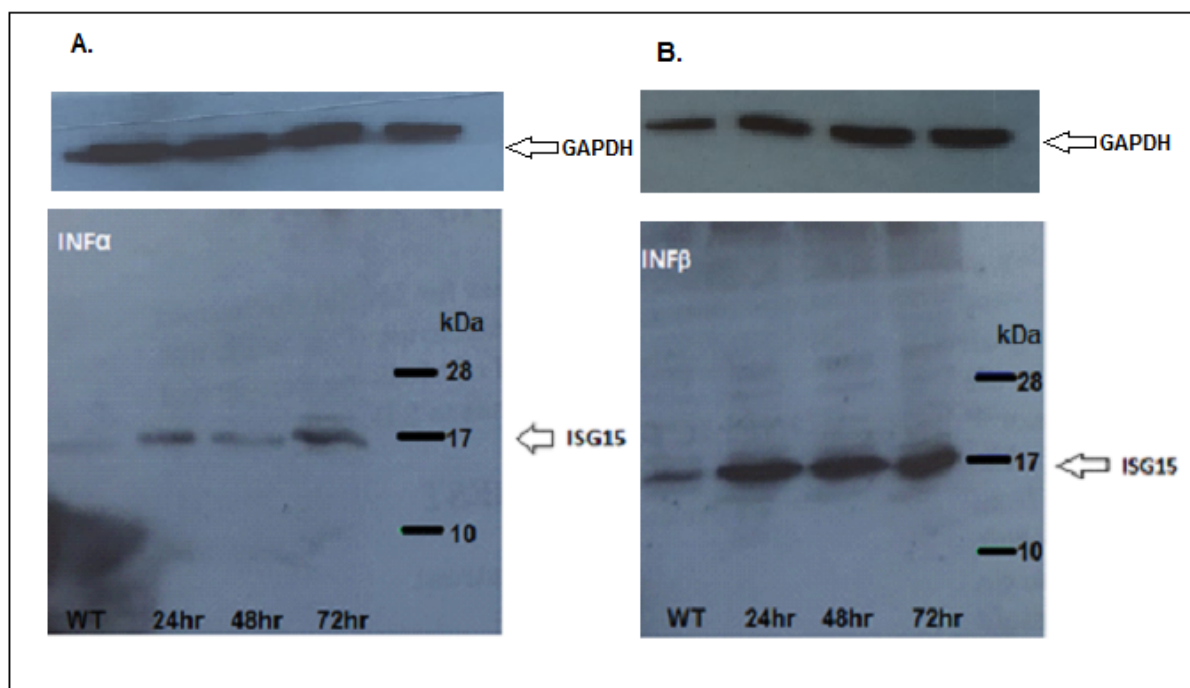


Figure 3.1.1: Western Blot Analysis of ISG15 Expression After INF Treatment
Image A shows the *INF α* induced ISG15 blot and Image B shows the *INF β* induced ISG15 blot

In Figure 3.1.1, Image A three P₁₀₀ plates of 80% confluent wild type siha cells were transfected with 1 μ g of *INF α* and left for 24, 48 and 72 hours respectively. In Figure 3.1.1, Image B three P₁₀₀ plates of 80% confluent wild type siha cells were transfected with 1 μ g of *INF β* and left for 24, 48 and 72 hours respectively. A P₁₀₀ plate of 95% confluency that had not been treated was also harvested and used as the wild-type control. These cells were lysed with 8M Urea buffer and 30 μ g of protein was loaded on a 15% SDS-PAGE gel. Primary antibody used was polyclonal rabbit anti-ISG15 at 1 in 2500. Secondary used was mouse anti-rabbit antibody at 1 in 1000. Blot was developed for 12 mins.

In Image A the bands are much more faint than those in Image B despite the fact that 30µg of protein was loaded on each gel. This suggests that ISG15 is more highly expressed in cells treated with $\text{INF}\beta$ than those treated with $\text{INF}\alpha$.

3.1.2 Timecourse of ISG15 Induction by $\text{INF}\beta$

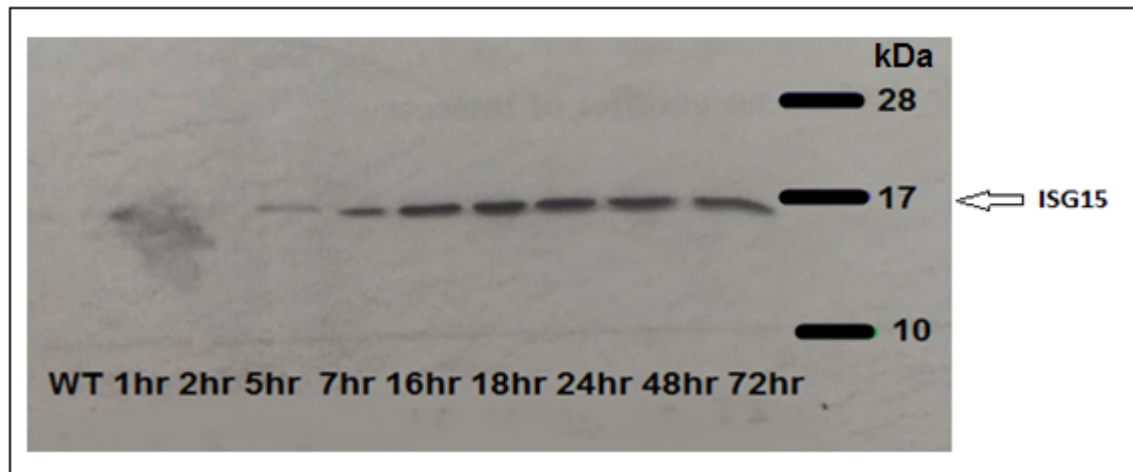


Figure 3.1.2: Western Blot Analysis of ISG15 Induction Timecourse

In Figure 3.1.2, 9 P₁₀₀ plates of 80% confluent wild type siha cells were transfected with 1µg of $\text{INF}\beta$ each and were left for 1, 2, 5, 7, 16, 18, 24, 48 and 72 hours respectively. A P₁₀₀ plate of 95% confluency that had not been treated was also harvested and used as the wild-type control. These cells were lysed with 8M Urea buffer and 30µg of protein was loaded on a 15% SDS-PAGE gel. Primary antibody used was polyclonal rabbit anti-ISG15 at 1 in 2500. Secondary used was mouse anti-rabbit antibody at 1 in 1000. Blot was developed for 2 minutes.

From this Figure 3.1.2 it is clear that the cells need to be transfected for at least 5 + hours for the ISG15 levels to be seen. The level of ISG15 increases steadily until 18 hours' post-transfection where it begins to level off. The bands become clear after 16 hours with 18 hours seeming the peak time as it shows the clearest band. No bands can be seen in the first 2 hours after transfection indicating that ISG15 induction begins between 2 and 5 hours after transfection.

3.1.3 $INF\beta$ induction of ISG15 Conjugation:

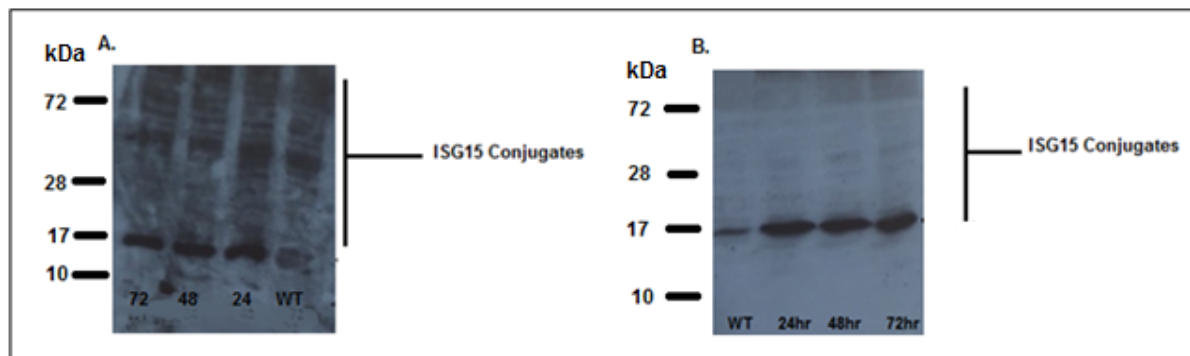


Figure 3.1.3: Western Blot Analysis of ISG15 Conjugates

Image A shows ISG15 and its conjugates after $INF\beta$ transfection and Image B shows ISG15 and transfection with $INF\beta$

In Figure 3.1.3, 9 P_{100} plates of 80% confluent wild type siha cells was transfected with $1\mu\text{g}$ of $INF\beta$ each and were left for 1, 2, 5, 7, 16, 18, 24, 48 and 72 hours respectively. A P_{100} plate of 95% confluency that had not been treated was also harvested and used as the wild-type control. These cells were lysed with 8M Urea buffer and $30\mu\text{g}$ of protein was loaded on a 15% SDS-PAGE gel. Primary antibody used was polyclonal rabbit anti-ISG15 at 1 in 2500. Secondary used was mouse anti-rabbit antibody at 1 in 1000. Blot 1 was developed for 20 minutes and blot 2 was developed for 2 minutes.

In Image A we can see multiple bands in each lane of the gel and these lanes are very dark and prominent. These bands are ISG15 conjugated to different proteins. In Image B we can also see multiple bands in each lane of the gel. These bands are much fainter however than those in image 1. No significant difference is seen between the different lanes in either gel. The bands are as prominent in the wild-type lane as they are in the $INF\beta$ treated lanes.

3.1.4 INF Induction of IG15 Pathway Members:

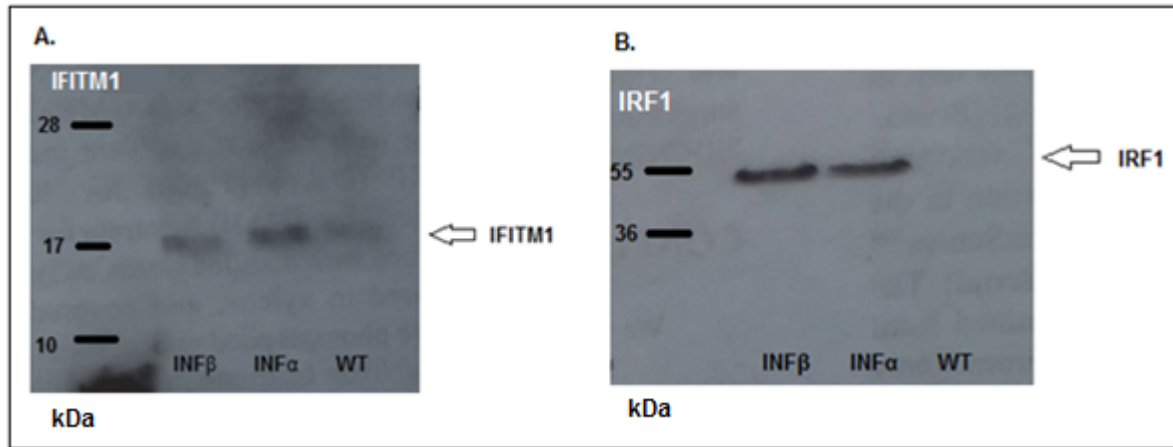


Figure 3.1.4: Western Blot Analysis of Interferon Induction of IRF1 and IFITM1
Image A shows IFITM1 following interferon induction. Image B shows IRF1 following interferon induction.

In Figure 3.1.4, a P₁₀₀ plate of 80% confluency was transfected with 1 μ g of INF α and was harvested after 24 hours. A separate P₁₀₀ plate of 80% confluency was transfected with 1 μ g of INF β and was harvested after 24 hours. A P₁₀₀ plate of 95% confluency that had not been treated was also harvested and used as the wild-type control. These cells were lysed with 8M Urea buffer and 30 μ g of protein was loaded on a 15% SDS-PAGE gel. In Image A, the primary antibody used was mouse anti-IRF1 at 1 in 1000. The secondary used was a rabbit anti-mouse antibody at 1 in 1000. In Image B, the primary antibody used was the Til anti-IFITM1 antibody at 1 in 1000. The secondary used was a rabbit anti-mouse antibody at 1 in 1000.

In both images of figure 3.1.4 bands can only be seen in the lanes that had been treated with interferons. No bands can be seen in the wild-type lanes.

3.2 CRISPR CAS Process

3.2.1 CRISPR Cas RNA targeting Exon 2:

The CRISPR RNA designed was transfected into Siha cells using GFP and attractene and sorted using FACS cell sorting. These cells were grown in 96-well plates. Clones were grown in separate wells of 6-well plates and were tested to see if they were positive CRISPR clones using PCR techniques and sequencing.

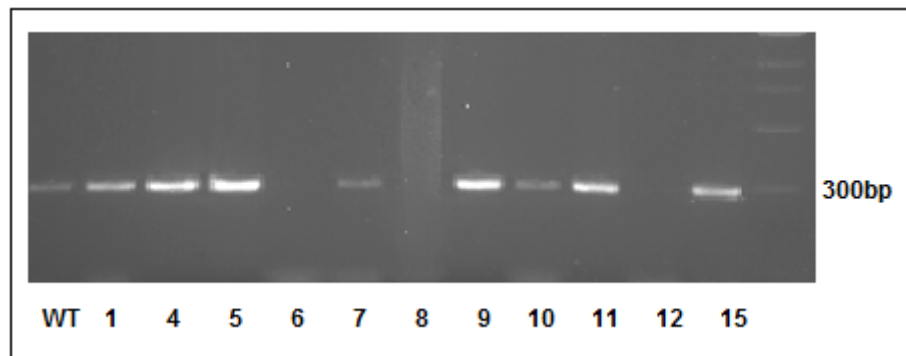


Figure 3.2.1: Agarose gel analysis of Exon 2 CRISPR PCR

The DNA is from cells which have been transfected with Exon 2 CRISPR RNA. The short PCR primers were used for these samples.

In Figure 3.2.1 the HF Phusion protocol was used and the resulting PCR product was run on a 1% Agarose TAE gel. The numbers on the lanes represent the names of the clones grown. We can see that there are multiple lanes that contain no bands. This could be due to low levels of DNA present or to the fact that the genome has been altered and that the primer region of the genome has been altered.

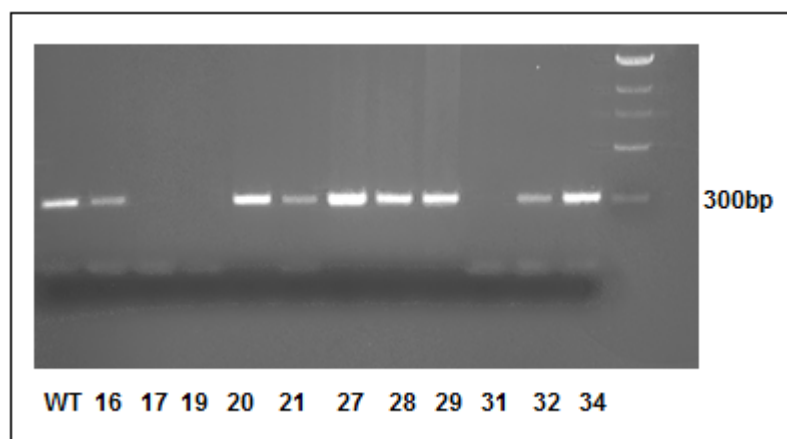


Figure 3.2.2: Agarose gel analysis of Exon 2 CRISPR PCR

The DNA is from cells which have been transfected with Exon 2 CRISPR RNA. The DNA is from cells which have been transfected with Exon 2 CRISPR RNA. The short PCR primers were used for these samples.

In Figure 3.2.2 the HF Phusion protocol was used and the resulting PCR product was run on a 1% Agarose TAE gel. Again, we can see that there are multiple lanes that contain no bands. This could be due to low levels of DNA present or to the fact that the genome has been altered and that the primer region of the genome has been altered.

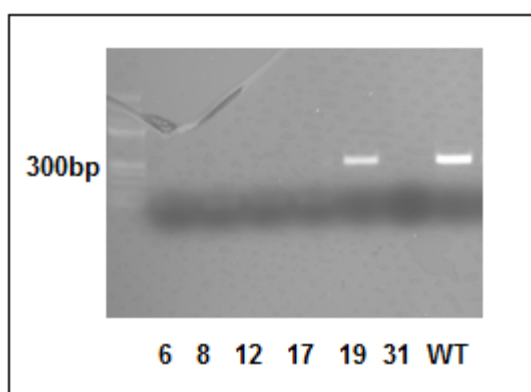


Figure 3.2.3: Agarose gel analysis of Exon 2 CRISPR PCR

The DNA is from cells which have been transfected with Exon 2 CRISPR RNA. The short PCR primers were used for these samples.

In Figure 3.2.3 the HF Phusion protocol was used and the resulting PCR product was run on a 1% Agarose TAE gel. The samples which showed no bands in the previous experiment were run again to make sure there were no mistakes. Sample 19 and the wild type control showed bands, meaning that the PCR worked this time and that the lack of bands in the previous experiment was likely an experimental error.

Following this experiment, the pure DNA of the seemingly negative samples were run on a 1% agarose TAE gel. The gel analysis showed that the DNA concentration of samples 8 and 19 were too low to be seen on a gel. This suggests the reason no bands were seen following the PCR reaction was due to the fact that there was negligible DNA present.



Figure 3.2.4: Agarose gel analysis of Exon 2 CRISPR PCR

P53 primers were used for these samples.

To ensure that the genome of the potentially positive CRISPR clones was intact and not completely disrupted, a PCR reaction was carried out using the HF Phusion protocol and the p53 primer. The resultant product was then run on a 1% Agarose TAE gel. From Figure 3.2.4 we can see this PCR protocol worked and that area of the p53 primers are still intact.

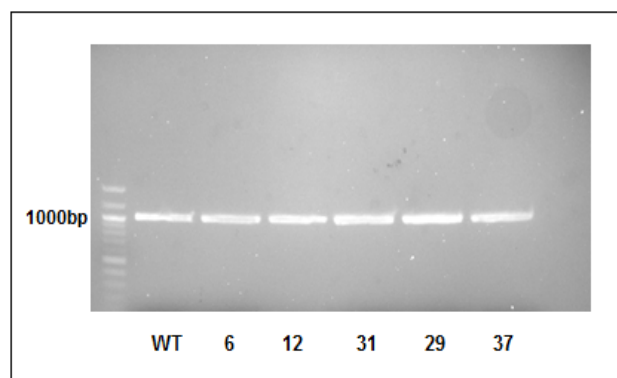


Figure 3.2.5: Agarose gel analysis of Exon 2 CRISPR PCR

The long PCR primers were used for these samples

In order to narrow down the area of the deletion, new primers were designed further apart from the original primers. This PCR reaction was carried out using the HF Phusion protocol and the resultant product was run on a 1% Agarose TAE gel. From this gel we can see that there are bands in every lane. This indicates that the area of the long primers is intact and the deletion is likely between these two long primers.

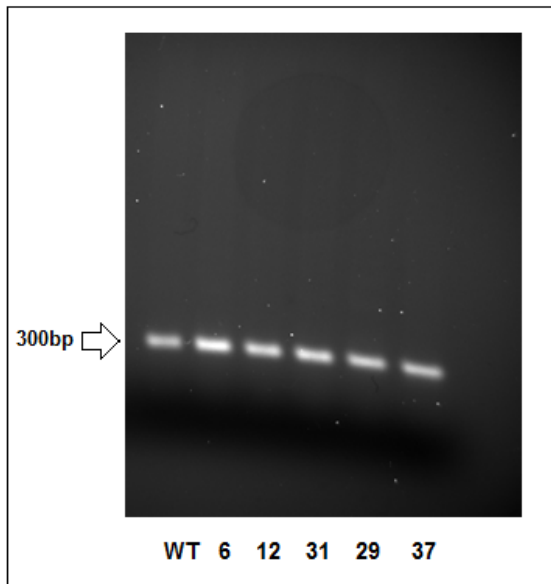


Figure 3.2.6: Agarose Gel Analysis of Exon 2 CRISPR PCR

The medium PCR Primers were used on these samples.

In an effort to further narrow down the area of the deletion, new primers were designed that were located between the long primers and the original short primers. This PCR reaction was carried out using the HF Phusion protocol and the resultant product was run on a 1% Agarose TAE gel. From Figure 3.2.6 we can see that there are bands in every lane. This indicates that the area of the medium primers is intact and the deletion is likely between these two medium primers.

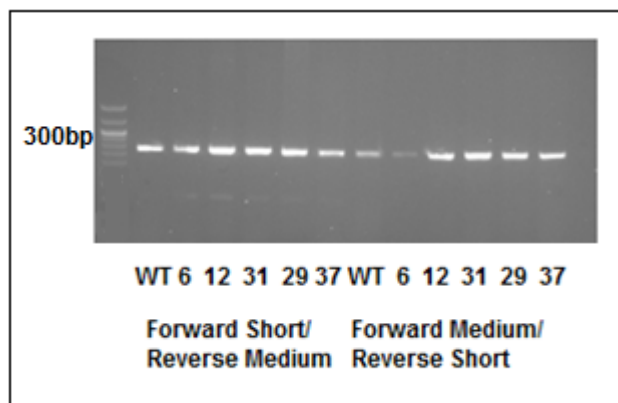


Figure 3.2.7: Agarose Gel Analysis of Exon 2 CRISPR PCR

In this PCR protocol the PCR primers were mixed in an attempt to try and narrow down the area of the suspected deletion

To further narrow down this area, the medium and short primers were mixed to test different areas for the presence of the deletion. This PCR reaction was carried out using the HF Phusion protocol and the resultant product was run on a 1% Agarose TAE gel. Again, in Figure 3.2.7 we can see that there are bands present in every lane. These primers identify very specific regions of the genome that are very close to the area identified by the short primers. The fact that there are bands present indicates that the results from the short primers may not be as accurate as previously believed. Only sequencing results can confirm this.

Sample 6:

Query	9	AGCAGCTCCNTGTCGGTGTCAAGCTG-AGGCGCAGATCACCAGAAGATCGGCGTGNAC	67
Sbjct	55	AGCAGCTCCATGTCGGTGTCAAGCTGAAGGCGCAGATCACCAGAAGATCGGCGTGAC	114
Query	68	GCCTTCCAGCAGCGTCTGGCTGTCCACCCGAGCGGTGTGGCGCTGAGGACAGGGTCCCC	127
Sbjct	115	GCCTTCCAGCAGCGTCTGGCTGTCCACCCGAGCGGTGTGGCGCTGAGGACAGGGTCCCC	174
Query	128	CTTGCCAGCCAGGGCTTGGGCCCCGGCAGCACGGTCTGCTGGTGGTGGACAAATGCGAC	187
Sbjct	175	CTTGCCAGCCAGGGCTTGGGCCCCGGCAGCACGGTCTGCTGGTGGTGGACAAATGCGAC	234
Query	188	GAACCTCTGAGCATCCTGGTGAGGAATAACAAGGGCCGACGACACCTACGAGGTGCGG	247
Sbjct	235	GAACCTCTGAGCATCCTGGTGAGGAATAACAAGGGCCGACGACACCTACGAGGTACGG	294
Query	248	CTGACG	253
Sbjct	295	CTGACG	300

Sample 12:

		GANCAGTCC-TGTCGGTGTCAAGCTG-AGGCGCANNATCACCAGAAGATCGGCGTGCA	65
		GAGCAGCTCCATGTCGGTGTCAAGCTGAAGGCGCAGATCACCAGAAGATCGGCGTGCA	113
		CGCCTTCCAGCAGCGTCTGGCTGTCCACCCGAGCGGTGTGGCGCTGAGGACAGGGTCCCC	125
		CGCCTTCCAGCAGCGTCTGGCTGTCCACCCGAGCGGTGTGGCGCTGAGGACAGGGTCCCC	173
		CCTTGCCAGCCAGGGCTTGGGCCCCGGCAGCACGGTCTGCTGGTGGTGGACAAATGCGA	185
		CCTTGCCAGCCAGGGCTTGGGCCCCGGCAGCACGGTCTGCTGGTGGTGGACAAATGCGA	233
		CGAACCTCTGAGCATCCTGGTGAGGAATAACAAGGGCCGACGACACCTACGAGGTGCG	245
		CGAACCTCTGAGCATCCTGGTGAGGAATAACAAGGGCCGACGACACCTACGAGGTACG	293
		GCTGACG	252
		GCTGACG	300

Sample 31:

Query	13	CAGTCCNTGTCGGTGTCAAGCTG-AGGCGCAGATCACCAGAAGATCGGCGTGACGC	71
Sbjct	57	CAGTCCATGTCGGTGTCAAGCTGAAGGCGCAGATCACCAGAAGATCGGCGTGACGC	116
Query	72	CTTCCAGCAGCGTCTGGCTGTCCACCCGAGCGGTGTGGCGCTGAGGACAGGGTCCCCCT	131
Sbjct	117	CTTCCAGCAGCGTCTGGCTGTCCACCCGAGCGGTGTGGCGCTGAGGACAGGGTCCCCCT	176
Query	132	TGCCAGCCAGGGCTTGGGCCCCGGCAGCACGGTCTGCTGGTGGTGGACAAATGCGACGA	191
Sbjct	177	TGCCAGCCAGGGCTTGGGCCCCGGCAGCACGGTCTGCTGGTGGTGGACAAATGCGACGA	236
Query	192	ACCTCTGAGCATCCTGGTGAGGAATAACAAGGGCCGACGACACCTACGAGGTGCGGT	251
Sbjct	237	ACCTCTGAGCATCCTGGTGAGGAATAACAAGGGCCGACGACACCTACGAGGTACGGT	296
Query	252	GACG	255
Sbjct	297	GACG	300

Figure 3.2.8: Sequencing Results for Sample 6, Sample 12 and Sample 31

Sequencing performed by the Cambridge branch of Science Biosource

To prepare the DNA for sequencing a PCR HF Phusion protocol was carried out at 50µl using the medium primers. 5µl of the resulting reaction was run on a gel to ensure that the PCR reaction had worked. Following this the remaining PCR reaction was prepared for sequencing using the Qiagen PCR Purification Kit. The DNA and primers were sent to Source Bioscience as per their instructions.

The sequencing results show that the PCR product is almost identical to the original genome sequence. Sample 6 and Sample 12 were 98% identical and Sample 31 was 99% identical to the original genome. This suggests that the genome has not been altered. The small differences between the CRISPR samples and the original genome is likely due to a sequencing error. There were multiple Ns in the CRISPR sequencing suggesting that not all the bases were correctly sequenced. It is highly probable that the differences are due to the sequencing shortcomings.

3.2.2 CRISPR Cas RNA targeting the C-Terminus:

As the results showed there the targeting of Exon 2 did not seem to work, the experiment was repeated. A new CRISPR RNA was also designed to target the C-Terminus. The CRISPR RNA for the C -Terminus and Exon 2 was designed was transfected into Siha cells using the electroporation technique and sorted using FACS cell sorting. These cells were grown in 96-well plates. Clones were grown in separate wells of 6-well plates and were tested to see if they were positive CRISPR clones using PCR techniques, western blots and sequencing.

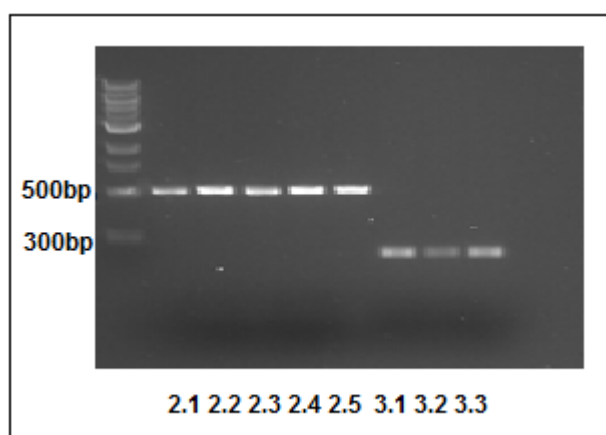


Figure 3.2.9: Agarose Gel Analysis of CRISPR Sample 2 and Sample 3 PCR

Sample 2 DNA are from the cells transfected with the C-Terminus CRISPR RNA. Sample 3 DNA are from the cells transfected with the Exon 2 CRISPR RNA.

The long primers were used for the Sample 2 PCR and the short primers were used for the Sample 3 PCR.

To determine whether the CRISPR RNA had altered the genome a PCR was run and then the results were analysed using agarose gels. In this figure the HF Phusion protocol was used and the resulting PCR product was run on a 1% Agarose TAE gel. The numbers on the lanes represent the names of clones grown. Bands can be seen in all lanes in this gel.

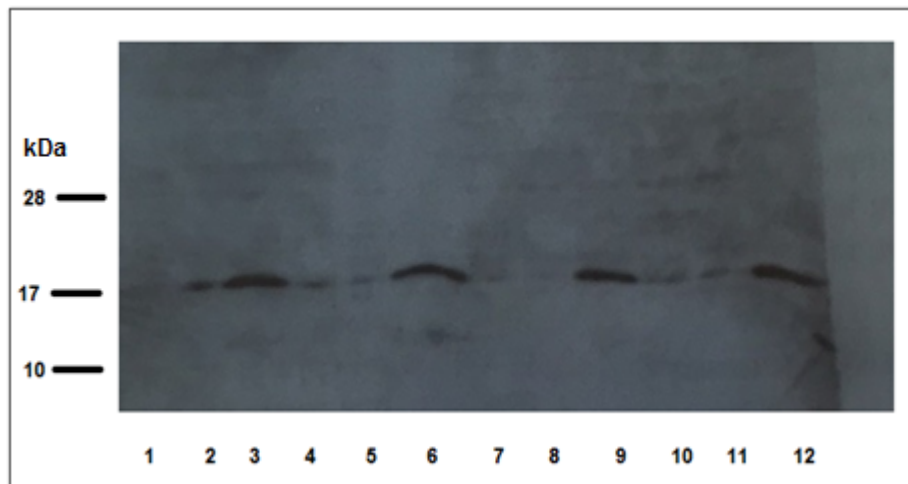


Figure 3.2.10: Western Blot Analysis of Wild-type, CRISPR and INF-treated CRISPR Cells

Lane 1: WT, Lane 2: 2.1, Lane 3: 2.1 INF, Lane 4: WT, Lane 5: 2.2, Lane 6: 2.2 INF, Lane 7: WT, Lane 8: 2.3, Lane 9: 2.3 INF, Lane 10: WT, Lane 11: 2.4, Lane 12: 2.4 INF.

In Figure 3.2.10, a P₁₀₀ plate of 80% confluency, of each CRISPR clone, was transfected with 1µg of INFβ and was harvested 24 hours later. A P₁₀₀ plate of 95% confluency of each clone was also harvested, untreated. A 95% confluent P₁₀₀ plate of wild-type Siha cells were also harvested to use as a control. These cells were lysed with 8M urea buffer and 30µg of protein was loaded on a 15% SDS-PAGE gel. Primary antibody used was polyclonal rabbit anti-ISG15 at 1 in 2500. Secondary used was mouse anti-rabbit at 1 in 1000. This blot was developed for 5 minutes.

In this Figure 3.2.10 we can see that bands are only present in the lanes which have been treated with interferons. No bands are seen in the wild-type lane or in the lanes with CRISPR cells that have not been treated with INFβ. As ISG15 protein can be seen after INF transfection, it suggests that the ISG15 gene has not been knocked down. Faint bands can be seen in Lane 3 (2.1 INF) and Lane 6 (2.2 INF) between the 10kDa and 17kDa markers, underneath the ISG15 band. These bands suggest that there may be a heterozygous deletion/mutation present.

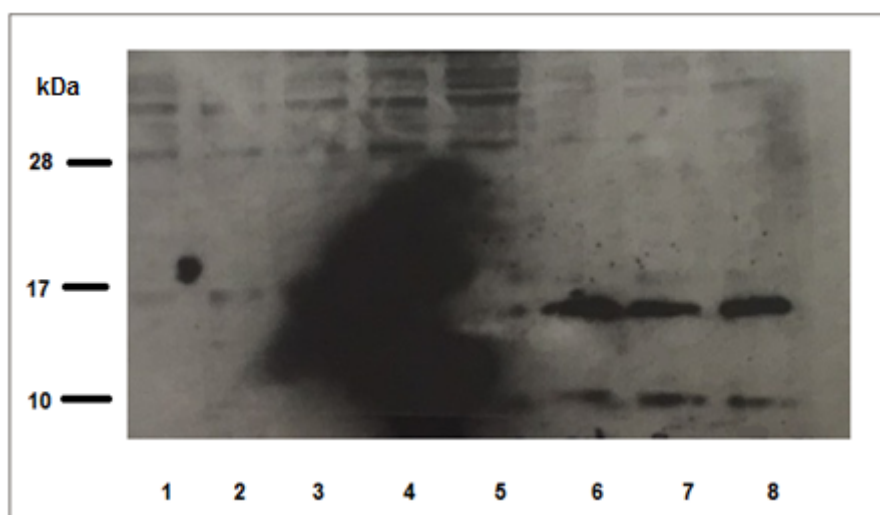


Figure 3.2.11: Western Blot Analysis of Wild-type, CRISPR and INF-treated CRISPR Cells
Lane 1: 2.5, Lane 2: 3.1, Lane 3: 3.2, Lane 4: 3.3, Lane 5: INF 2.5, Lane 6: INF 3.1, Lane 7: INF 3.2, Lane 8:
INF 3.3

A P₁₀₀ plate of 80% confluency, of each cell line, was transfected with 1µg of INFβ and was harvested 24 hours later. A P₁₀₀ plate of 95% confluency of each cell line was also harvested, untreated. A 95% confluent P₁₀₀ plate of wild-type Siha cells were also harvested to use as a control. These cells were lysed with 8M urea buffer and 30µg of protein was loaded on a 15% SDS-PAGE gel. Primary antibody used was polyclonal rabbit anti-ISG15 at 1 in 2500. Secondary used was mouse anti-rabbit at 1 in 1000. This blot was developed for 5 minutes.

In this Figure 3.2.11, we can see that bands are only present in the lanes which have been treated with interferons. No bands are seen in the wild-type lanes or in the lanes with CRISPR cells that have not been treated with INFβ. As ISG15 protein can be seen after INF transfection, it suggests that the ISG15 gene has not been knocked down. In this image we can see that there are clear bands around the 10kDa marker, below the ISG15 protein. Again, these bands suggest that there may be a heterozygous deletion/mutation present. Based on these results, the CRISPR cells show a potential alteration in the genome.

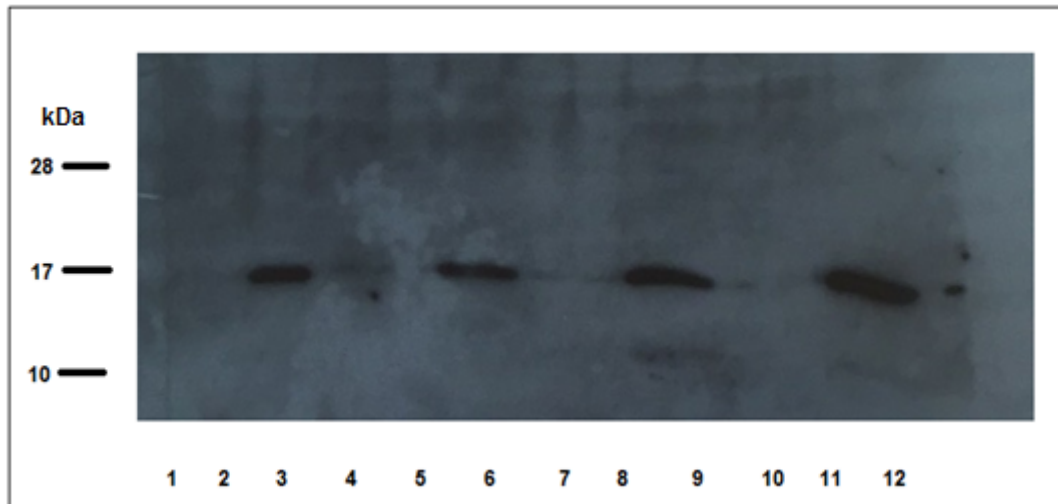


Figure 3.2.12: Western Blot Analysis of Wild-type, CRISPR and INF-treated CRISPR Cells
Lane 1: WT, Lane 2: 2.5, Lane 3: 2.5 INF, Lane 4: WT, Lane 5: 3.1, Lane 6: 3.1 INF, Lane 7: WT, Lane 8: 3.2, Lane 9: 3.2 INF, Lane 10: WT, Lane 11: 3.3, Lane 12: 3.3 INF.

This is a repeat experiment using the same cell lysate as figure 3.2.11. The cell lysate had been snap frozen using liquid nitrogen and was stored overnight at -80°C. The lysate was thawed on ice and was used at the same concentration that had been determined via Bradford reagent the previous day. 30µg of protein was loaded on a 15% SDS-PAGE gel. Primary antibody used was polyclonal rabbit anti-ISG15 at 1 in 2500. Secondary used was mouse anti-rabbit at 1 in 1000. This blot was developed for 5 minutes.

Again in Figure 3.2.12 we can see that bands can only be seen in the lanes treated with INF. There are also faint bands on this gel below the ISG15 protein, around the 10kDa marker. In the previous figure these bands can be seen in all lanes treated with INF yet in this gel the bands can only be seen in Lane 9 (3.2 INF) and Lane 12 (3.3 INF). It is not clear why the bands can no longer be seen in 2.5 INF and 3.1 INF. The protein may have started to deteriorate after being frozen and thawed or it may be that the first image had been an experimental mistake. Sequencing results will be necessary to determine if the genome of these cells has been altered. Sequencing was attempted with these samples yet due to DNA contamination, clear results were not obtained.

3.3 Single Chain Purification of ISG15:

3.3.1 Plasmid Induction Single Chain Protocol A

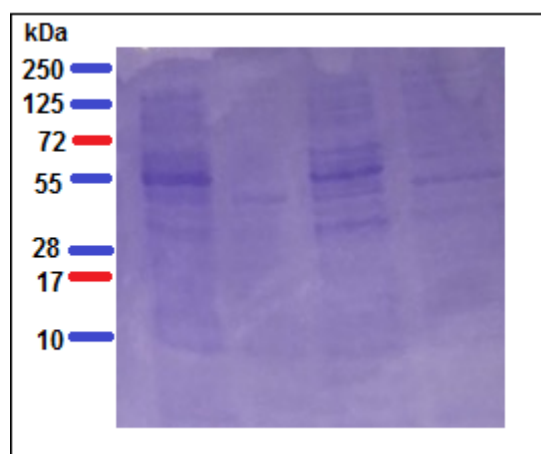


Figure 3.3.1: Commassie Gel Analysis of Bacterial Culture Samples

Lane 1: Whole Cell Before Induction **Lane 2:** Whole Cell After Induction **Lane 3:** Cell Lysate Before Induction **Lane 4:** Cell Lysate After Induction

A 1ml aliquot was taken of the bacterial culture before induction with 1M IPTG and a 1ml aliquot was taken after induction when the OD level was 0.445. These samples were spun down at 13,200rpm for 15 mins at 4°C. The pellets were resuspended in varying amounts of PBS depending on their OD600 measurements. These samples were sonicated at 10.0 amp in the cold room. The sample taken before induction was sonicated 6x5 seconds with 30 second intervals on ice. The sample taken after induction was sonicated 10x5 seconds with 30 second intervals on ice. After sonication 50µl of both samples were taken and labelled Whole Cell Before Induction and Whole Cell After Induction respectively. The remainder of the samples were spun down at 6000rpm for 20 minutes at 4°C. The supernatant was removed and placed in tubes labelled Cell Lysate Before Induction and Cell Lysate After Induction respectively. 10µl of each sample was added to 10µl of Loading Dye 0.25M DTT. These sample were boiled at 95°C for 3 mins and loaded onto a 15% SDS-PAGE gel. The gel was run for 45mins at 170V and was stained in 15ml of Commassie Brilliant Blue Solution and then destained using destaining solution.

The plasmid, if expressed, should be roughly 26kDa in size. No distinct bands can be seen at this size. This may indicate that the plasmid is not present or it may indicate that it is present in a dimer and may be one of the heavier bands present.

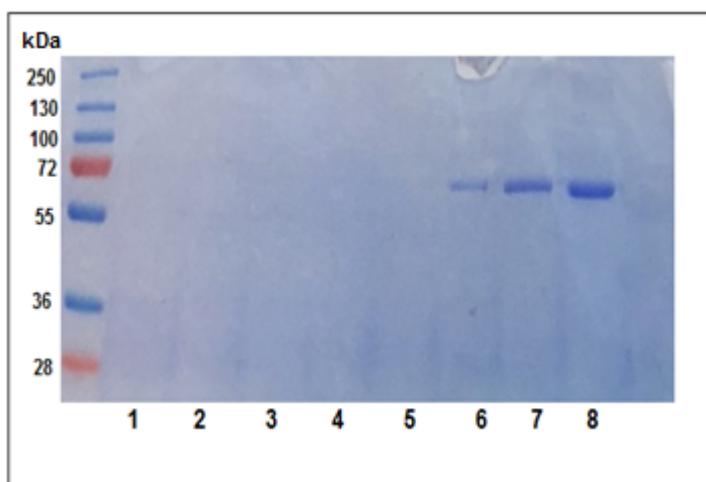


Figure 3.3.2: Compassie Gel Analysis of Single Chain Elutes and Bradford Standards

Lane 1: Elute 1, **Lane 2:** Elute 2, **Lane 3:** Elute 3, **Lane 4:** Elute 4, **Lane 5:** Elute 5, **Lane 6:** 0.1µg BSA standard, **Lane 7:** 0.2µg BSA standard, **Lane 8:** 0.5µg BSA standard

These elutes were obtained using protocol A. As we can see there are no bands in the elute lanes. If the single chain was present in the elutes we would see a band around the 26kDa. In lanes 6, 7 and 8 of Figure 3.3.2 we see bands around the 65kDa mark, these are the BSA standards. The BSA standards are used to give an indication of the quantity of the single chain if the single chain is present.

10µl were added into each lane (5µl of protein and 5µl of 0.25M DTT Loading Dye). The gel was stained in 15ml of Compassie Brilliant Blue Solution and then destained using destaining solution.

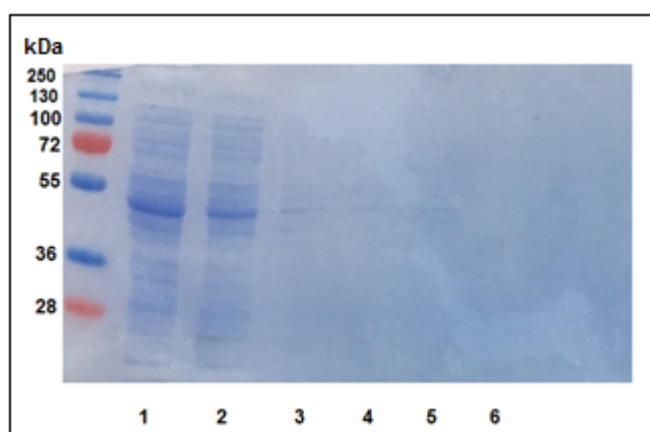


Figure 3.3.3: Compassie Gel Analysis of Protocol A Single Chain Washes and Flow-Through

Lane 1: Flow-through **Lane 2:** Wash 1
Lane 3: Wash 2 **Lane 4:** Wash 3 **Lane 5:** Wash 4 **Lane 6:** Wash 5

In Figure 3.3.3 10µl was added to each lane (5µl of protein and 5µl of 0.25M DTT Loading Dye). The gel was stained using 15ml of Compassie Brilliant Blue Solution and then destained using destaining solution. The washes and flow-through were obtained from Protocol A. These

samples are run on a gel to ensure the single chain hasn't been washed through in the washes which can sometimes occur.

3.3.2 Plasmid Induction and Purification of Single Chain Protocol B and C

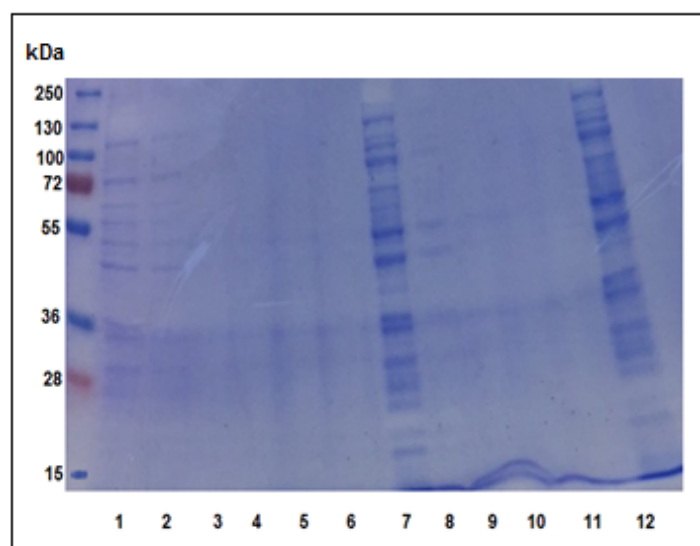


Figure 3.3.4: Compassie Gel Analysis of Protocol B and Protocol C Single-Chain Washes and Flow-Throughs

Lanes 1-6 were from Protocol B and Lanes 7-12 were from Protocol C

Lane 1: Flow-through **Lane 2:** Wash 1 **Lane 3:** Wash 2 **Lane 4:** Wash 3 **Lane 5:** Wash 4 **Lane 6:** Wash 5 **Lane 7:** Wash 1 **Lane 8:** Wash 2 **Lane 9:** Wash 3 **Lane 10:** Wash 4 **Lane 11:** Wash 5 **Lane 12:** Flow-through

10µl was added in each lane. (5µl of protein and 5µl of 0.2M DTT Loading Dye). The samples were incubated at 37°C for 10 minutes prior to being run on the gel. The gel was then stained with Brilliant Blue Compassie Stain and destained using destaining solution. From Figure 3.3.4 we can see that there is very little protein in the washes and flow-through from Protocol B. This combined with the fact that no protein was found in the elution's suggests that the plasmid was not efficiently induced and the single chain is likely not present. In the flow-throughs and first wash of protocol C we can see an abundance of protein. Some of the single-chain seems to have been eluted into the flow-through and first wash as a band can be seen around the 36kDa mark. This would be roughly the size of the single chain in its dimer form. The single chain was also found in the elutes however so it was not entirely lost in the washes.

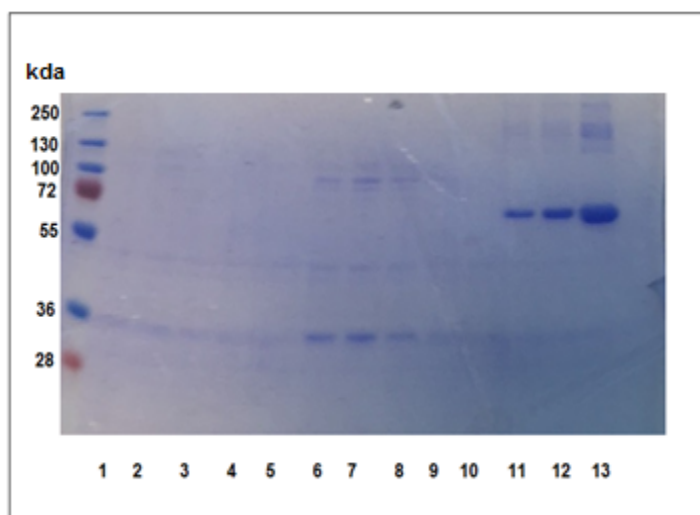


Figure 3.3.5: Coomassie Gel Analysis of Protocol B and Protocol C Elutes and BSA Standards

Lanes 1-5: Protocol B Elutes 1-5, **Lanes 6-10:** Protocol C Elutes 1-5, **Lanes 11-13:** BSA standards 0.1 μ g, 0.2 μ g and 0.5 μ g.

The elutes from protocol B show only very faint bands around the 36kDa marker. The faintness of these bands compared to the BSA standards shows that their concentrations are likely too low to be functional. Elutes 1, 2 and 3 of protocol C show more distinct bands around the 36kDa marker. These bands are likely the single chain protein. Based on the faintness of the elute bands to the BSA standard bands shows that the concentration of the elutes are likely to be low yet it is worth testing their affinity.

3.3.3 Testing the Affinity of the Purified Single Chain

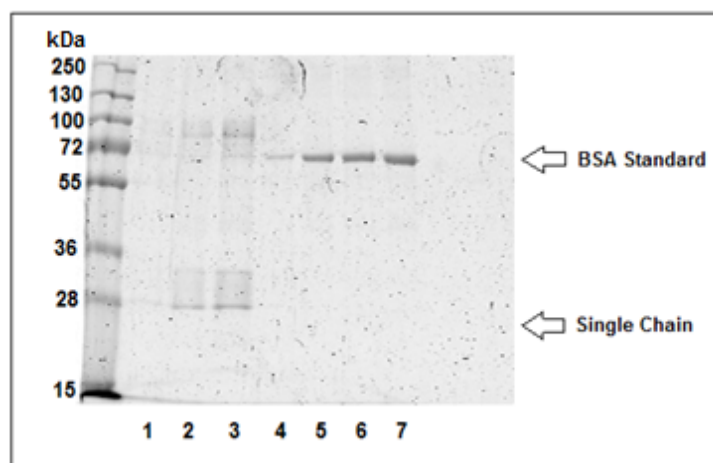


Figure 3.3.6: Quantification Analysis Purified Single Chain

Lane 1: Protocol B Elute 3, **Lane 2:** Protocol C Elute 1, **Lane 3:** Protocol C Elute 2 **Lane 4:** 0.01 μ g BSA, **Lane 5:** 0.03 μ g BSA, **Lane 6:** 0.05 μ g BSA, **Lane 7:** 0.1 μ g BSA

The concentration of the single chain is far too low to test using common protein quantification techniques such as using a Bradford reagent. To test the concentration of the single chain, quantitative analysis on a commassie gel was performed. The gel was stained with 15ml of Brilliant Blue Commassie Stain and destained thoroughly using destaining solution. The gel was then scanned using Odessey Gel scanner and was analysed using Image Studio Lite Western Gel Analyser. The intensity levels of the BSA standards were plotted on graph against their concentrations. The concentrations of the elutes were then calculated using the equation of the linear trend line of the graph. From the graph in Figure 3.3.7 we can see that the concentration of protocol B elute 3 is too low to analyse.

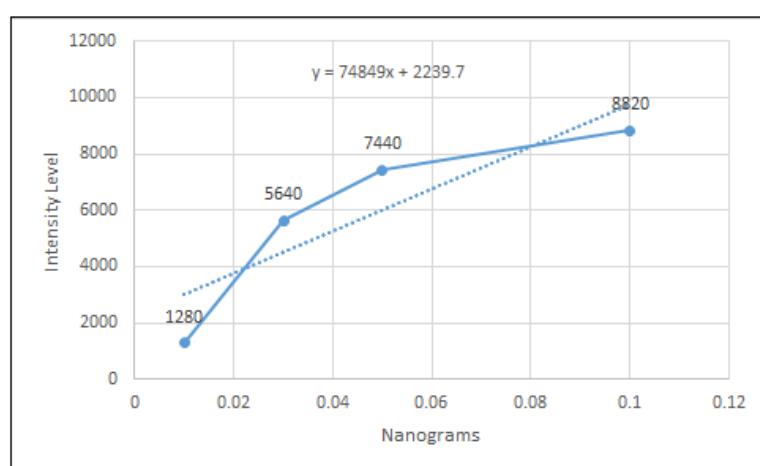


Figure 3.3.7: Excel Graph of Single Chain Concentration Analysis

Filling the intensity level of Protocol C elute 3, 3090, into the equation $y = 74849x + 2239.7$ a concentration of 0.012 was calculated.

When the intensity level of Protocol C elute 2, 1620, was filled into the equation, a negative number was obtained. The concentration of Elute 2 was too low to be determined in this manner. Using purely visual analysis of Figure 3.3.6, the concentration of elute 1 was determined to be half that of elute 2, roughly 0.006.

To test the affinity of the single chain antibody, an ELISA was performed. 50ng of pure protein was added to the first 10 wells. Sodium carbonate solution was added to the next 10 wells as a control. Titrations of the single chains were used as the primary antibody. The concentrations calculated were based on the original single chain concentrations which were later thought to be three times more concentrate. The secondary used was Protein A HRP at 1 in 5000.

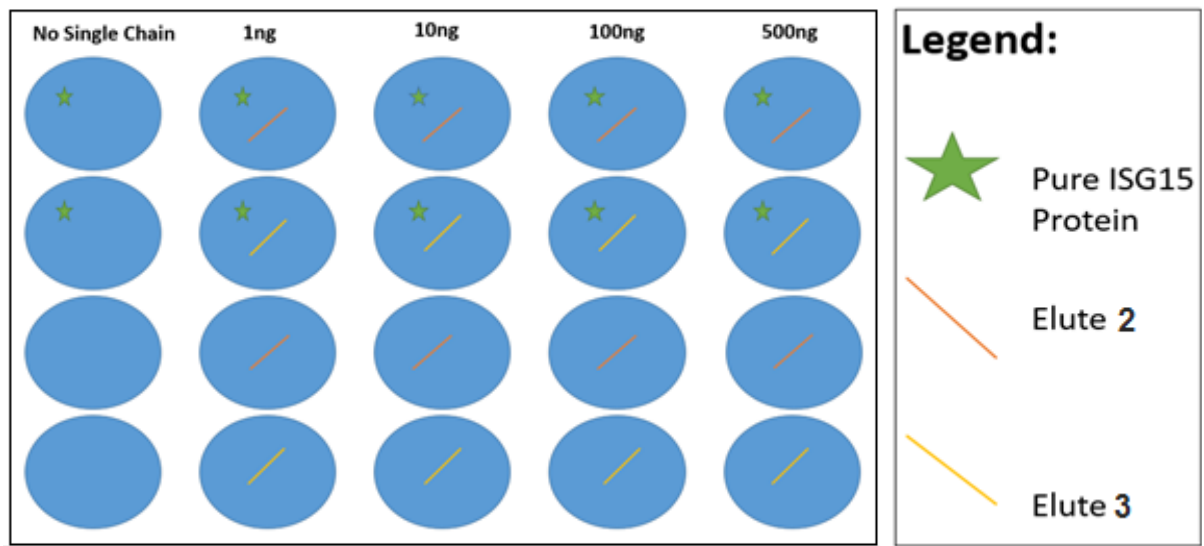


Figure 3.3.8 Illustration of ELISA Plate Layout

The plate was developed using ECL and the ELISA Microplate Reader. The results were plotted on an Excel graph.

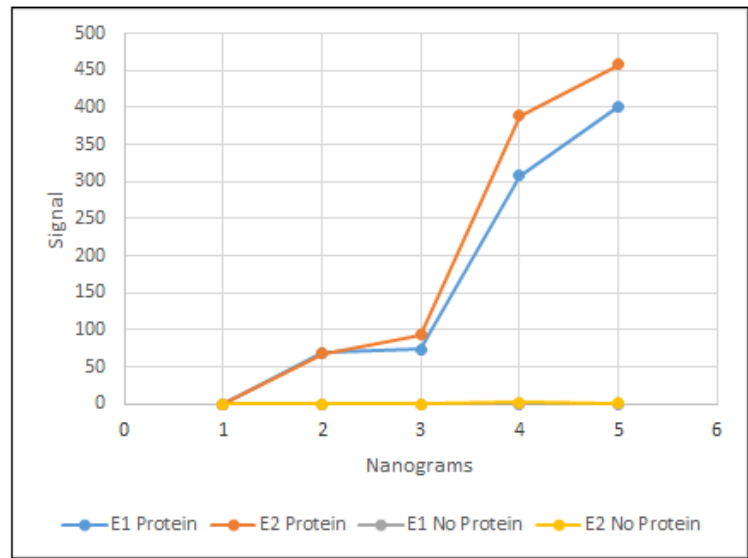


Figure 3.3.9: Excel Graph of ELISA Results

Based on the graph in Figure 3.3.9 we can see that even in low quantities the single chain has the ability to bind to the pure protein. The signal increases dramatically with the increase in concentration.

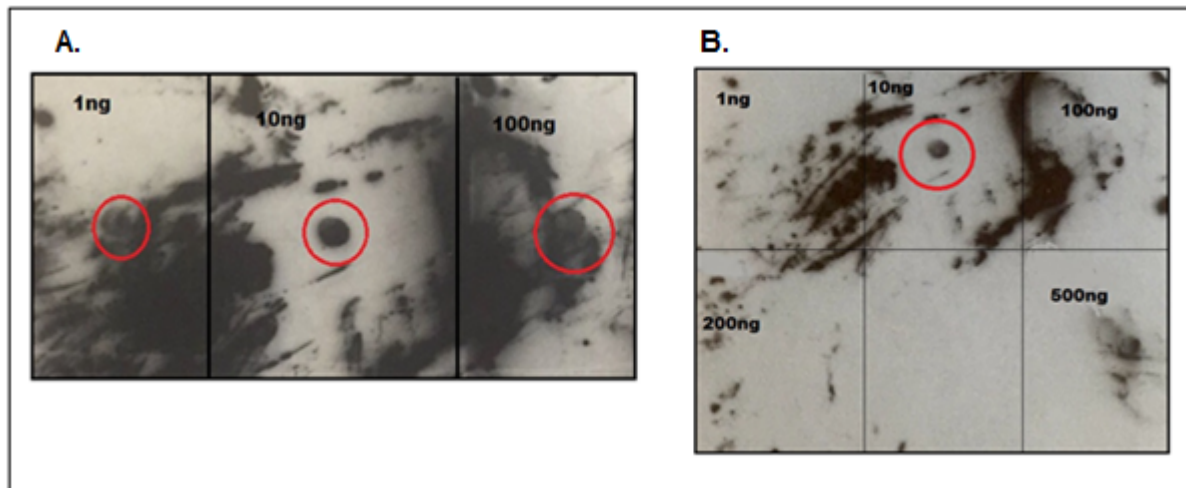


Figure 3.3.10: Dot Blot Analysis of ISG15 using ScFv as Primary Antibody

Image A was the first blot taken. Blot was developed for 5 minutes. Image B was the second blot taken. Blot was developed for 12 minutes.

2 μ l of protein was added in each square. A titration of concentrations of pure ISG15 protein was used (1ng – 500ng). 80 μ l of Elute 2 in 2.5ml of 5% PBST-milk was used as the primary antibody. This was the equivalent of approximately 2mg/ml. Primary antibody was left on overnight at 4°C. The secondary used was Protein A HRP at 1 in 5000. Blot was developed for 4 minutes.

In Image A of Figure 3.3.10, dots can be seen in the squares with concentrations of 1ng, 10ng and 100ng. These dots are unclear however due to the dirtiness of the blot. When the blot was washed again, a dot could only be seen in the 10ng square. The vigorous washings could have stripped some of the proteins from the blot.

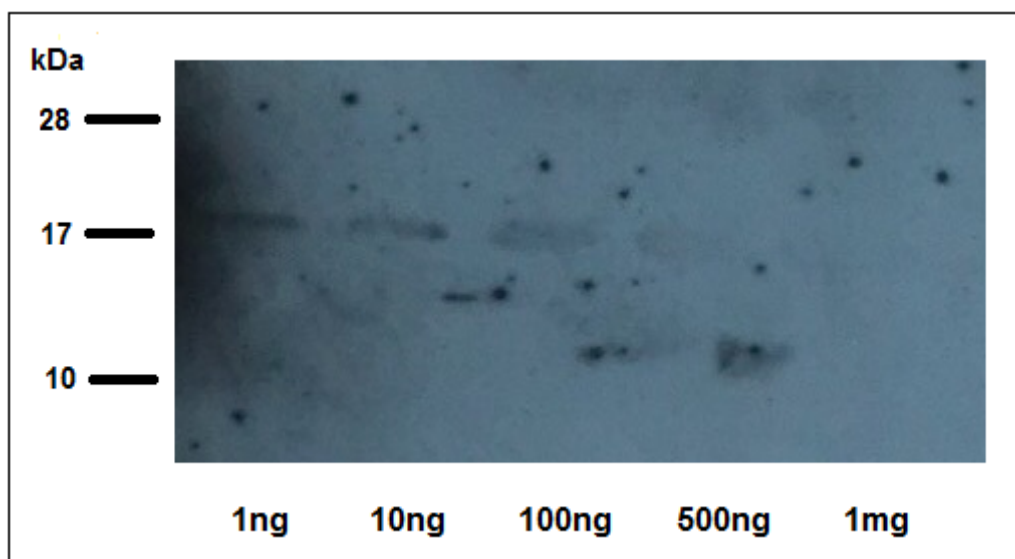


Figure 3.3.11: Western Blot Analysis of ISG15 using ScFv as Primary Antibody

10 μ l was added to each well (5 μ l of protein and 5 μ l of 0.2M DTT Loading Dye). A titration of concentrations of pure ISG15 protein was used. 80 μ l of Elute 2 in 2.5ml of 5% PBST-milk was used as the primary antibody. This was the equivalent of approximately 2mg/ml. Primary antibody was left on overnight at 4°C. The secondary used was Protein A HRP at 1 in 5000. Blot was developed for 6 minutes.

Clear bands can be seen in the first 3 lanes, 1ng, 10ng and 100ng, of the gel in Figure 3.3.11. Despite the higher concentrations, no bands can be seen in the last two lanes, 100ng and 500ng.

Chapter 4

Discussion:

4.1 Interferon Induction of the ISG15 Pathway

In Figure 3.1.1 it is clear that there is a stronger induction of protein GAPDH was used as a loading control. The induction of ISG15 by type 1 interferons has been well documented throughout the literature (Bektas et al. 2008; Dieterich & Relman 2011; Forster 2012) yet few papers differentiate between the inducing potential of interferon α and interferon β . This experiment ISG15 when cells are transfected with $\text{INF}\beta$ than when cells are transfected with $\text{INF}\alpha$. The bands in figure 3.1.1 Image B are significantly darker to those bands in figure 3.1.1 Image A. In both blots 30ng of protein was added to each lane and the blots were developed for the same length of time. The housekeeping suggests that $\text{INF}\beta$ has a stronger induction potential than $\text{INF}\alpha$.

Although $\text{INF}\alpha$ and $\text{INF}\beta$ are both type 1 interferon and have similar structures and functions, they are not completely identical. For example, their transcription methods are entirely different. The transcription of interferon β is accomplished through the collaboration of multiple transcription factors recruited to several specific DNA-binding sites in the VRE of the $\text{INF}\beta$ promoter. (Juang et al. 1998) Less is known about the transcription of the $\text{INF}\alpha$ gene. IRF-E and AF-1 are involved however as over-lapping cis-acting elements. The IRF-E site is crucial for transcriptional activity. The subtle differences between the two interferons could alter their induction potential.

The differences between the two gels in Figure 3.1.1 could also be due to experimental error. The loading controls show the same amount of protein was added to each gel. It also showed that there was no transferring error. The experiment should have been repeated to consolidate the result yet due to lack of $\text{INF}\alpha$ this was not possible. Based on this result, $\text{INF}\beta$ was used in the following the experiments to induce ISG15.

In Figure 3.1.2 the length of time needed for ISG15 induction was investigated. Zhao et al has described transfecting cells for 24 hours in order for ISG15 to be seen clearly on a Western blot using commercial antibodies. (Zhao et al. 2005) In order to optimise experimental procedure, it is necessary to determine the peak time of ISG15 induction after interferon transfection. To do this, Siha cells were transfected with $\text{INF}\beta$ for varying length of time. When

the resulting cell lysate was run on an SDS-PAGE gel, the time at which ISG15 starts to be induced can be clearly seen. In the Figure 3.1.2 we can see that ISG15 begins to be induced between 2 and 5 hours' post-transfection as no bands can be seen at the 2-hour mark yet a faint band can be seen at the 5-hour mark. This suggests that cells need to be transfected for at least 5 hours with INF β in order for ISG15 to be seen on a blot. The level of ISG15 then increases steadily until the 24-hour mark where it begins to level off. There is no significant difference between the bands at 18, 24, 48 and 72 hours. Peak transfection time seems to be from 18 hours onwards with little to no difference between the ISG15 induction levels at the latter time points.

As previously stated, IG15 has the ability to bind to multiple proteins intracellularly. This process is known as ISGylation and the proteins involved play critical roles in multiple vital cellular processes. In order to determine the roles of ISG15 conjugates, they must first be identified. In this experiment, cells were transfected for 24, 48 and 72 hours respectively. In Image B, Figure 3.1.3 only very faint bands can be seen above the ISG15 band. This blot had been developed for 2 minutes. No difference can be seen between the lanes that had been transfected with interferons. In Image A, Figure 3.1.3 we can see multiple dark bands above the ISG15 band. These bands are the ISG15 conjugates. Again in this image we can see that there is no significant difference between the lanes that had been treated with interferons. These dark bands can even be seen in the wild-type lane. These results suggest the length of transfection is not as important in conjugation identification as the length of time developing with ECL.

IRF1 and IFITM1 are integral components of the ISG15 pathway and they are both induced by Type 1 interferons. In Figure 3.1.4, in both images, bands can only be seen in lanes treated with type 1 interferons. This shows that cells must be interferon stimulated in order for IRF1 or IFITM1 to be detected. Therefore, both IRF1 and IFITM1 must not be present in untreated cells or, present in such small amounts that they cannot be detected using commercial antibodies.

Based on these results, it is clear that Type-1 interferons are crucial inducers of the ISG15 pathway. Both interferon α and interferon β are capable of significantly inducing members of this pathway, including ISG15, IRF1 and IFITM1, as long as they are transfected for a minimum of 18 hours.

4.2 CRISPR CAS Process

4.2.1 CRISPR Cas RNA targeting Exon 2:

The aim of targeting the genome with the CRISPR RNA was to induce a knock-down of our target gene, ISG15. There are multiple ways to test if the gene has been knocked down or if the genome has been altered in any way. The first test carried out as part of this experiment was to design PCR primers that flank the CRISPR RNA. Over 30 CRISPR clones were grown and tested. In Figure 3.2.1, the first set of clones were tested. From this figure we see that most of the lanes have clear bands around the 300bp mark. This is the correct size for the distance of the two primers. Three lanes have no bands. This could be due to an alteration in the genome such as a deletion. Alternatively, it could be an experimental mistake such as not enough DNA in the PCR mix. The HF Phusion PCR protocol has worked however as the wild-type lane shows a band. This proves the annealing temperature and time is correct. In Figure 3.2.2 the second set of clones were tested. Again this figure shows that all but three lanes have bands around the 300bp. A band is present in the wild-type lane showing the PCR protocol was run correctly. The lanes without bands are the potential positive CRISPR clones.

In Figure 3.2.3 the potential positive clones were run again to alleviate concern of potential experimental mistakes. Again in the gel in Figure 3.2.3, a band is seen in the wild-type lane showing the PCR protocol has worked. A band is also seen in the clone 19 lane indicating the PCR has worked this time. To ensure that the DNA of the potentially positive clones was not compromised, the pure DNA was run on an agarose gel. A picture of the gel is not available but the results showed that the DNA in clone 8 and clone 17 was too low to be seen on a gel. Strong bands were seen in the wild-type, clone 6, clone 12 and clone 31 lanes. Therefore, clone 6, clone 12 and clone 31 are highly likely to be positive CRISPR clones.

To test if the genome of the potential positive CRISPR clones are still intact, the HF Phusion protocol was run using p53 primers. In Figure 3.2.4 lanes can be seen around the 500bp mark in all lanes. This is approximately the size of the distance between the two primers. The bands present indicate the genome is still intact.

To try and identify the area of the supposed deletion, new primers were designed that were much further apart than the original primers. In Figure 3.2.5, the products from the PCR using the long primers were run on an agarose gel. Bands are seen in all lanes, including the wild-type lane indicating that the PCR has worked. This result indicates that the supposed deletion is between these two primers.

To further narrow down the area of the deletion, new primers were designed that are located between the long primers and the original primers. In the gel in Figure 3.2.6 bands can be seen in all lanes. The PCR protocol has worked as there is a band present in the wild-type lane. The deletion is, again, likely to be between these two primers.

A smaller area is always preferential for sequencing as it increases efficiency. To identify the exact location of the deletion, the medium and short primers were mixed. In Figure 3.2.7 bands are present in all lanes in the gel. As the area between the two mixed primers is very similar to the area between the two original primers, it is very surprising that no deletion is seen. These results suggest that perhaps the first set of PCR results were an anomaly despite being repeated.

In order to determine for sure if a deletion was present in these clones, they were sent for sequencing. The sequencing results are shown in Figure 3.2.8. The sequencing results were aligned to the original genome sequence using NCBI nucleotide BLAST function. The results conclusively prove that there is no deletion in the genome of the clones. Sample 6 and Sample 12 were 98% identical and Sample 31 was 99% identical to the original genome. The small differences between the CRISPR samples and the original genome is likely due to a sequencing error. There were multiple Ns in the CRISPR sequencing suggesting that not all the bases were correctly sequenced. It is highly probable that the differences are due to the sequencing shortcomings.

Despite the initial positive PCR results the CRISPR Cas process did not work in this case. This could be due to a multitude of factors. The CRISPR Cas process is still a very new technique that has not been completely optimised. New techniques and programmes such as the MIT CRISPR RNA design program are starting to revolutionise the creation of CRISPR RNA by enabling the creation of highly specific oligos that have very low off-targets. The transfection and sorting process is also flawed. The cells are transfected with GFP and sorted based on the presumptive idea that cells which contain the GFP also contain the plasmid. This is not always the case of course. In fact, only approximately one third of cells sorted will contain the plasmid.

Altering the genome of the cell can also have unknown effects. Many genes play critical roles in key cellular functions. Knocking out these genes can be fatal in these cells which will lead to the lack of positive clones found. ISG15 plays a crucial role in anti-viral immunity (Pitha-Rowe & Pitha 2007) as well as multiple other roles when conjugated to a variety of proteins. As no other papers have described the effects of a knockdown of ISG15 it is still unknown as to whether an ISG15 knockdown is fatal.

4.2.2 CRISPR Cas RNA targeting the C-Terminus and Exon 2:

As the attempt to knockdown ISG15 by targeting the beginning of the coding exon of the gene, exon 2, failed, new CRISPR RNAs were designed to target the C-terminus of the gene.

In Figure 3.2.9 the HF Phusion PCR protocol was run using DNA from the CRISPR clones. From here we can see that bands are present in all lanes suggesting that the DNA obtained from the CRISPR cells has not be altered. However, a very small deletion may not be seen in a gel like this. Running a PCR is just the first step in trying to identify a genome alteration. PCR analysis works well to identify large alterations. It is very difficult to identify smaller deletions in this manner as the bands can still appear to be similar sized.

Following these results, the clones were tested using Western Blot analysis. As we have previously identified the optimum conditions for identifying ISG15, we applied these principals to testing the clones. From the gel in Figure 3.2.10 we can see bands present in the lanes that have been treated with $\text{INF}\beta$. No bands can be seen in the lanes which have not been treated with the type-1 interferon. The fact that bands can be seen in the clones transfected with $\text{INF}\beta$ suggests that the gene has not been knocked down. In Figure 3.2.10 very faint bands can be seen around the 10kDa marker in the interferon-treated lanes of 2.1 and 2.2. This band is too small to be the ISG15 protein. Identifying a protein this size suggests that there may be a heterozygous mutation/deletion present and that this band could perhaps be a truncated form of the ISG15 protein. The second set of CRISPR clones were run using the same method. This gel can be seen in Figure 3.2.11. In this gel we can see that band can only be seen in lanes where the clones had been transfected with type-1 interferons. Again this suggests that the ISG15 gene is intact. Also in this gel we see the distinct bands of a smaller protein is present around the 10kDa mark in the 3.2 and 3.3 clones which had been treated with $\text{INF}\beta$. These bands may also be present in the 2.5 and 3.1 $\text{INF}\beta$ clones but due to the dirtiness of the blot it cannot be seen.

As this blot was not very clear, the same lysate was run again using the same protocol. The results, which can be seen in Figure 3.2.12, show a slightly different picture. In this gel the smaller band can only be seen in lane 9 which is clone 3.2 treated with $\text{INF}\beta$. There are a number of reasons why the smaller band may no longer be present in the other interferon treated lanes. Firstly, an experimental error could have occurred in the gel in Figure 3.2.11 such as a movement of the film while transferring of a movement of the film while developing. If either one of these mistakes were made, the bands around the 10kDa marker are likely to be the same

ISG15 band just lower down. Alternatively, the cell lysate may have started to degrade. Despite snap-freezing in liquid nitrogen and storing at -80°C overnight, the lysate may have become unstable and the smaller protein may no longer be present.

In order to comprehensively determine if there was a genomic deletion or alteration present, all of the sample clones were sent for sequencing. Due to a contamination of the DNA of the clones, it was not possible to sequence the DNA. As there were time constraints at the end of the project it was not possible to extract new DNA from the clones. As the sequencing results could neither deny or confirm the alteration of the genome it is still unknown as to whether a deletion is present.

Although it has not been confirmed, the results from these experiment suggest that the genome has been altered. The same CRISPR plasmid for Exon 2 was used in both experiments yet quite different results were seen. The only other alteration in experimental procedure was the use of electroporation instead of attractene to transfect the plasmid. This may not be the reason behind the differences in the results yet it is possible that electroporation increases the odds of a more effective transfection.

4.3 Single Chain Purification of ISG15:

The single chain purification protocol A was based on the protocol designed by Mariana Cristina De Sa Cardoso Dos Santos during her MRes in the lab of Ted Hupp. Throughout the protocol, samples were taken at every step in order to determine where and when the single chain has been induced before starting the purification step. The samples taken from protocol A can be seen in the gel Figure 3.1.1. All of the lanes contain bands, yet the bands in lanes before induction are more prominent and more frequent than the bands in the lanes after induction. If the single chain is present, it will likely be in the after induction lanes and a clear band should be seen around the 36kDa mark. No bands of this size can be seen in any of the lanes. In fact, most of the protein after induction seems to be degraded.

Despite this, an attempt to purify any single chain present was carried out. Figure 3.3.2 and Figure 3.3.3 show the commassie stained gels of the elutes and the washes. From Figure 3.3.3 we can that some of the protein has remained in the flow-through and in wash 1. Whether the single chain is in this remains unclear. As no protein was seen in the elutes it is possible that the single chain was pulled out in the flow-through and in the first wash. It is also possible that there was no protein present as an exact band was not seen in the bacterial culture gel.

Based on these observations it was decided to repeat the experiment and alter the protocol.

In Protocol A the plasmid was left to induce overnight at 30°C. From the gel in Figure 3.3.1 we can see that very little protein is present after the long induction time, especially any smaller proteins. Based on these observations it was concluded that the long incubation time was a major problem in the protocol and so in Protocol B the incubation time was cut to 6 hours. Protocol C was carried out at the same time with an incubation time of just three hours. From the gel in Figure 3.3.4, we can see that very little protein is present in the flow-through and wash lanes. This could be a positive sign as it means the single chain protein has not been eluted through the washes. However, the lack of protein in the washes and flow through coupled with the lack of elutes seen in Figure 3.3.5, it suggests that the single chain has not been induced. No clear bands can be seen around the 36kDa mark in any of the lanes in Protocol B, either in the elutes or washes and flow through.

In the Protocol C lanes in Figure 3.3.4, multiple distinct bands can be seen in the flow-through and wash 1 lanes. A band can be seen in these lanes around the 36kDa marker indicating that there is a strong possibility of the single chain being present. Due to the fact the bands can be seen in the elute lanes in Figure 3.3.5, the entirety of the single chain was not lost in the flow through and washes. The bands in Elute 1, Elute 2 and Elute 3 are distinctive and around the 36kDa marker. The faintness of the bands compared to the BSA lanes suggests the concentration is very low. Protocol C, however, has worked.

As the plasmid is quite small, a lower incubation time seems to work more efficiently. The longer the plasmid is incubated for the more protein is degraded. Larger proteins can withstand the incubation period better than the smaller more soluble proteins. It was necessary to understand this in order to move forward with the experiment.

Before the affinity of the antibodies can be tested, their concentrations must be determined. As the concentrations of the single chains are too low to be determined using a Bradford reagent test, other less accurate methods must be pursued.

In Figure 3.3.7 the intensity levels of the BSA standard bands from the gel in Figure 3.3.6 were mapped on an excel graph. Using the equation of the graph, the concentration of the Elute 1 was deemed to be negligible. The concentration of Elute 2 was also too low to be accurately determined using this method. The concentration of Elute 3 was calculated to be roughly 0.012.

This method is not entirely accurate however. The BSA standards are approximately three times the size of the single chain protein and therefore absorb three times as much loading dye, increasing the intensity level by a factor of three. Based on this the concentration of elute 1 would be approximately 0.018 and the concentration of elute 2 is likely 0.036.

As described in Figure 3.3.8 a variety of concentrations were used as the primary antibody. These concentrations were calculated based on the much lower original single chain concentrations. The results of the ELISA were plotted on an Excel graph as shown in Figure 3.3.9. from this graph we can see that the single chain has a very high affinity for pure ISG15, even in low concentrations. No protein was added to two lanes as a control.

To further test the ability of the single-chain, a dot blot was performed. A dot blot is a simplified version of the western blot which eliminates the need for gel electrophoresis. The molecules are not separated in this procedure but since only a pure protein was loaded this was not necessary. In Image A Figure 3.3.10, although the blot is messy, dot can be seen in squares with concentrations of 1ng, 10ng and 100ng. Although the other two squares had higher concentrations of 200ng and 500ng, no dots could be seen. The blot in Image A Figure 3.3.10 was so dirty that it is possible the dots in the 200ng square and 500ng square were obstructed. The blot was washed again thoroughly with PBST in an attempt to clean the blot further, despite having performed multiple washes before the addition of ECL. When the blot was washed again and developed, a dot was only seen in the 10 ng square. The washing has likely washed off the protein. The antibody is likely very sticky as the blot was quite dirty despite multiple washes. A lower concentration of primary antibody would probably have given a cleaner blot. In this blot we also see failure of the single chain to bind to higher concentrations of proteins. While this may be just an experimental mistake, it may also indicate an inability of the single chain to bind when saturated with protein.

The ELISA protocol and the dot blot test the ability of the single chain to bind to the pure form of ISG15. When a protein is run on an SDS-PAGE gel however the protein becomes denatured and binding site of the protein may be altered. To investigate if this is the case and to identify the amount of loaded protein necessary for the single chain binding, a titration of pure ISG15 concentrations were loaded. The single chain was used as the primary antibody as at 2mg/ml. This concentration was calculated using the more accurate, higher concentration of the single chain elute 3. From the gel in Figure 3.3.11 we can see that the single chain has the ability to bind to even very low concentrations of protein. No clear bands can be seen in the lanes with

higher concentrations of protein. This anomaly is likely due to an error in transferring. Despite the denaturing of the ISG15 on the gel, the single chain antibody was still capable of efficiently binding to the protein.

Single Chain purification is a highly effective method of creating a specific antibody for a certain gene. By optimising the process for inducing and purifying the single chain, it will be possible to recreate this experiment and produce large quantities of a single chain antibody with a high affinity for the ISG15 protein.

Future Directions:

During the course of these experiments a number of positive results were obtained. These results however opened the door for a wide variety of future experiments to be carried out. Identifying the proteins involved in ISGylation would greatly increase our understanding of the roles of ISG15. As it known how to induce ISGylation, samples could be sent for mass spectrometry and the proteins involved could be quantified.

If time constraints were not an issue, the DNA of the CRISPR clones would be extracted from fresh samples and they would be resent for sequencing in order to identify any genomic alterations. If the sequencing results showed positive CRISPR clones, a multitude of experiments could be carried out. These would include treating the clones with Camptothecin as literature has stated that cells which lack ISG15 show decreased sensitivity to CPTs. This could be compared to the wild type cell lines which should have a higher sensitivity.

The single chain purification elutes could be further analysed to determine their concentrations. Having the correct concentrations would increase the accuracy of the experiments and their results and would allow for a more in depth interpretation of their affinity. The single chain could then be used for any experiments which require an ISG15 antibody. The protocol could also be altered and tested to try and further optimise the plasmid induction. Protocol C, the only working protocol, was run at 37°C which is quite a high temperature for plasmid induction. It was thought that the shorter incubation time required a higher temperature however inducing the plasmid at the lower temperature, even for the shorter time, is likely to be effective.

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